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THE PYOGENIC STREPTOCOCCI OF LANCEFIELD GROUP C AND GROUP G AS PATHOGENS IN MAN

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DECLARATION

I, Androulla Efstratiou, declare that whilst registered as a candidate for the degree of Doctor of Philosophy at the City of London Polytechnic, was not registered for any other award of the CNAA or of a University. The work undertaken during this period was carried out by myself, with guidance from Dr G.Colman of the Central Public Health Laboratory and Mrs C.Gaylarde of the City of London Polytechnic. The studies undertaken during this period include the attendance of seminars and lectures held at the Central Public Health Laboratory. Seminars have been given at the Central Public Health Laboratory and City of London Polytechnic. Poster demonstrations have been presented to groups of medical microbiologists at the Central Public Health Laboratory. A workshop held by LKB on isoelectric focusing and protein separation was attended (April 1984). Oral and poster communications have been presented at the Pathological Society of Great Britain and Ireland (January 1985 and January 1987). Abstracts for two presentations have been accepted for the Xth International Lancefield Symposium on Streptococci and Streptococcal Diseases, Cologne, Germany (September 1987). Four papers have been published.

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ABSTRACT

A collection of Lancefield group C and group G streptococci was examined for biochemical or serological properties that could provide the basis for their subdivision beyond species and serological group.

Substrates specific for 80 different enzymes were available and the profiles of activity among 100 selected strains showed some relationship to the sources of the strains. Nine tests were selected as key reactions and may be useful for defining nine species within these serological groups.

T-protein antigens, similar to those found on *Streptococcus pyogenes* (Lancefield group A) were detected on 87% of human isolates of group C and 82% of strains of group G. A total of 20 different T-protein antigens were detected and could be extracted from the bacterial cell by trypsin. In this state they had a molecular weight of 28,000 daltons and were immunogenic.

When strains were tested for survival in fresh human blood approximately one half of the throat isolates and all cultures from septicaemia multiplied. Whole cell vaccines prepared against strains resistant to phagocytosis produced precipitating antisera that had the properties of M-typing sera. Seven M antigens were recognised among strains of group C and seven among group G. Pepsin extracts from these strains contained material of 34,000 daltons that reacted with M-typing sera and 28,000 daltons that were identified with T-typing sera.

The serotyping scheme was applied to a total of 1547 random isolates and 600 strains from clusters of infections in which the evidence supplied by the clinical laboratory was consistent with an outbreak of infection. The results suggested serotyping would be a useful laboratory-based method applicable to epidemiological studies.

ABBREVIATIONS

ADB : Anti-deoxyribonuclease B
AGN : Acute glomerulonephritis
AHT : Anti-hyaluronidase titre
ASO : Anti-streptolysin O
BCIP : 5-bromo-4-chloro-3-indoyl phosphate toluidine salt
C : Cross-linking
C' : Complement
CFT : Complement fixation test
CIE : Countercurrent immunoelectrophoresis
CDSC : Communicable Disease Surveillance Centre
DMF : N-dimethyl-formamide
DNA : Deoxyribonucleic acid
ELISA : Enzyme linked immunoassay
h : Hours
HAP : Hydroxylapatite
HMW : High molecular weight
HRP : Horseradish peroxidase
IEF : Iso-electric focusing
LMW : Low molecular weight
LTA : Lipotechoic acid
mA : Milliamps
MAP : M-associated protein
MBC : Minimal bactericidal concentration
MHD : Minimum haemolytic dose
MIC : Minimal inhibitory concentration
min : Minutes
NBT : p-nitro blue tetrazolium chloride
NCTC : National Collection of Type Cultures
NSAP : Nephritis strain-associated protein
OD : Optical density
PAG : Polyacrylamide gel
PAGE : Polyacrylamide gel electrophoresis
Rf : Relative migration value
RNA : Ribonucleic acid
sec : Seconds
SDS : Sodium dodecyl sulphate
T : Gel concentration
TBS : Tris-buffered saline
TRIS : Tris-hydroxymethylmethylamine
TTBS : Tween-20-TBS
v : Volts

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1. INTRODUCTION

1.1. THE STREPTOCOCCI

The term 'streptococcos' was first used by Billroth (1874) to describe chain-forming coccoid-shaped bacteria which he noted in wounds and discharges from the animal body. He also noted the presence of the 'streptococcos' in about one-half of the cases of erysipelas which he examined. The terms 'streptococcos' and 'streptococcus' were subsequently used by various authors to designate a particular kind of cell congregation and were not used in the generic sense. This organism was recognised as an infectious agent by Fehleison in 1882. However, the generic name Streptococcus was first used by Rosenbach (1884) to describe a coccus, growing in chains, which he had isolated from suppurative lesions in man. To this organism he gave the name Streptococcus pyogenes. Since then, numerous methods of classifying these organisms have been proposed. Several species produce very characteristic changes in media containing blood and those producing clear zones of lysis around the colony (β-haemolytic) were first designated Streptococcus haemolyticus by Schottmüller in 1903. The name Streptococcus mitior seu viridans was proposed to describe those colonies which were surrounded by zones of green colourisation on blood agar media. These observations formed the basis of a routine identification procedure still in current use.

The first attempt at a definitive classification of streptococci was that of Andrewes and Horder (1906). These workers used the fermentation of several sugars, reduction of neutral red, growth

characteristics in milk and gelatin, morphological observations and virulence experiments in animals. Their sole test of virulence was animal inoculation, by the injection of mice and rabbits with whole cells of streptococci. Agglutination tests were performed against a series of other streptococci and on this basis they established that considerable differences do exist but at that time were not sufficiently defined to aid in the classification of these organisms. However, on the basis of the biochemical tests previously mentioned, they distinguished five groups of streptococci, S.pyogenes, S.salivarius, S.anginosus, S.faecalis and the Pneumococcus sp.

The next major contribution to streptococcal classification was made by Orla-Jensen (1919). He used a greater variety of tests including fermentation characteristics, tolerance to heat and sodium chloride, temperature limits of growth and cellular morphology. He recognised nine species namely, S.lactis, S.cremoris, S.mastitidis, S.thermophilus, S.bovis, S.inulinaceus, S.faecium, S.glycerinaceus and S.liquefaciens.

In 1937, Sherman subdivided the streptococci into four main divisions (1) the pyogenic streptococci; these are usually β-haemolytic, are not heat-resistant and have a polysaccharide Lancefield antigen; (2) the enterococci; these vary in haemolysis, are somewhat heat-resistant and have the Lancefield group D antigen; (3) the lactic streptococci; these grow at a low temperature and are less tolerant to other extreme environmental

conditions and (4) the 'viridans' streptococci; which are seldom β-haemolytic and grow at 45°C. Most workers however, have had difficulty in repeating the last observation. However, two of the categories of streptococci designated by Sherman (1937) might be transferred to different genera. The lactic streptococci (S.lactis and related streptococci) could form the genus Lactococcus (Schleifer et al. 1985). The transfer to the new genus would be predominantly based on nucleic acid hybridisation studies. The second category of streptococci that could be transferred to a new genus are the enterococci, which would be placed into the genus Enterococcus (Schleifer and Kilpper-Bälz 1984) again based on nucleic acid studies and cell-wall analysis.

Sherman (1937), established three defined biochemical groups within the Lancefield group C streptococci, S.equi as the cause of 'strangles' in horses, the 'animal pyogenes' or the 'animal C' type and the 'human C' streptococcus which was said to be the characteristic group C form obtained from human sources. They could be distinguished on the basis of their fermentation patterns for lactose, trehalose and sorbitol. He described Lancefield group G streptococci as two type forms. The type I form referred to the large colony variety established by Lancefield and Hare (1935) and the type II as the 'minute' colony form of Long and Bliss (1934) now designated S.milleri.

The genus Streptococcus belongs to the family called Lactobacilliaceae. They are Gram-positive, spherical or oval

cells arranged in chains of varying length; each cell is approximately 1.0 µm in diameter, non-motile (some have been described as motile (Hashimoto et al. 1985) and some may be capsulated. The first claim for motility in streptococci was in 1902 by Ellis and was found in some enterococci, isolated from dairy products and faeces. The majority of streptococci are aerobes (or facultative anaerobes), but there are species that are microaerophilic. Glucose is fermented with the production of lactic acid almost exclusively.

Most of the haemolytic streptococci may be subdivided into a number of broad groups determined by the chemical nature of the carbohydrate contained in their cell wall (Hitchcock 1924, Lancefield 1928a, 1933). The antigen upon which grouping of the haemolytic streptococci is based was first described by Hitchcock (1924) as a 'residue antigen' and was believed by him to be common to most haemolytic streptococci. This view prevaled until its group-specific nature was discovered by Lancefield (1933). In 1933 Lancefield classified streptococci serologically by their carbohydrate antigens. The serological test was based on a precipitation reaction between acid-extracted antigens and specific antisera prepared in rabbits. Lancefield's technique is still widely used and has been modified very little over the last fifty years. There are now 21 serological groups with sequential letters A to W in the alphabet (except I and J) and provisional groups X, Y and Z (Facklam and Edwards 1979). Amongst the β-haemolytic streptococci which have a Lancefield group antigen,

group A (Streptococcus pyogenes) is the major human pathogen and causes many diseases from local inflammatory lesions such as sore throat or pyoderma to fatal septicaemia.

Lancefield group C and group G streptococci are also widely distributed. Veterinarians have noted their occurrence in a variety of animals. They are also known as an occasional cause of serious disease in humans. These organisms exist as normal flora in the pharynx (Forrer and Ellner 1979), on the skin (Sherman 1937), in the gastrointestinal (Barnham 1983) and female genital tracts (Christensen et al. 1974) and infections are often associated with these sites. The commonest sites of infection seem to be in wounds and on the skin. Asymptomatic existence in the upper respiratory tract may be followed by pharyngitis and lymphadenitis. Lancefield group G streptococci may be carried asymptomatically in the female genital tract (Christensen et al. 1974, Morris and Morris 1967) resulting in an increase of infections amongst newborn infants (Lancefield and Hare 1935, Dyson and Read 1981). Serious diseases due to representatives of these groups include septicaemia (Mohr et al. 1979, Auckenthaler et al. 1983, Finch and Aveline 1984), endocarditis (Blair and Martin 1978, Goldberg et al. 1985), septic arthritis (Gaunt and Seal 1986), pneumonia, meningitis, pharyngitis, otitis media and cellulitis. They have also been suggested as the presumptive cause of hospital outbreaks but there has been no previous serotyping scheme available to test this possibility.

Table 1

Differentiation of beta-haemolytic S.milleri from other beta-haemolytic streptococci

Streptococci	Enzyme hydrolysis of *PYR	Enzyme hydrolysis of †β-GUR	‡VP test	Hydrolysis of arginine	Acid produced from ribose
Group A					
S.milleri	-	-	+	+	-
S.pyogenes	+	-	-	+	-
Group C					
S.milleri	-	-	+	+	-
S.equisimilis	-	+	-	+	+
S.equi	-	+	-	+	-
S.dysgalactiae	-	+	-	+	-
S.zooepidemicus	-	+	-	+	-
Group G					
S.milleri	-	-	+	+	-
Streptococcus sp.	-	+	-	+	+
Group F					
S.milleri	-	-	+	+	-

* PYR = pyrrolydonylarylamidase
† β-GUR = β-glucuronidase

‡ V.P. = detection of acetoin (Vogues-Proskauer test)

(Bucher and von Graeventiz 1984)

Streptococcus milleri (Guthof 1956) is considered to be an important cause of purulent disease in humans (Parker and Ball 1976). Although most of these strains are non-haemolytic, approximately 25% are beta-haemolytic and may cross react with the Lancefield group A, C, F or G antigen (Ball and Parker 1979). Beta-haemolytic S.milleri with the group A, C or G antigen may be distinguished from S.pyogenes (group A), S.equisimilis (group C in humans), and the large-colony group G streptococci, biochemically and morphologically (Table 1), (Bucher and von Graeventiz 1984, Lawrence et al. 1985). Recent studies have revealed the presence of Fc receptors on S.equisimilis strains from cases of pharyngitis. However, these receptors were absent from S.milleri (with the group C antigen) of healthy individuals (Lebrun et al. 1986). These surface structures have been implicated as possible contributors to the pathogenicity of the organism (Burova et al. 1982).

1.2 STREPTOCOCCAL DISEASE IN MAN

Many different streptococci are present on the human body surfaces and nearly all of them have been known to cause disease. The streptococcal diseases of man are various and range from highly communicable epidemics to opportunistic infections in hospital patients. In most, the local septic lesion is the sole manifestation of disease, but in some there is a tendency for the streptococcus to spread through connective tissue, along the lymphatics or into the bloodstream. There seems to be a tendency

for certain species to cause infections at particular sites. For example acute tonsillitis can be caused by the streptococci of Lancefield group A (Fry 1983).

The study undertaken by Hope-Simpson (1981) indicated that of 1437 episodes of sore throat, group A streptococci were recovered from only 242 (16.7%). Hence, most sore throats are not caused by streptococci and the cause is usually never found.

Skin lesions are of two sorts: namely impetigo, which is a superficial crusted lesion or pyoderma at the site of minor injuries. From these, streptococci sometimes spread to deeper tissues. The other is an acute inflammation of wounds or burns without crusting but usually with pus formation and a tendency to spread through the lymphatics. Streptococcal impetigo is usually caused by group A strains but strains of groups C and G have also been implicated (Reid et al. 1985).

Bacterial factors responsible for the virulence of streptococci in man may be looked upon as determinants for 1) invasiveness, the ability to gain entry to tissues and to spread from the initial focus of infection, 2) toxicity, 3) localisation with the production of inflammatory lesions in particular organs, 4) immunogenicity in diseases with an immunological compound.

A cell wall antigen, the M protein, is the antiphagocytic molecule which is presumably responsible for the virulence of the group A

streptococcus in systemic disease. The results obtained in laboratory experiments in mice (Dochez et al. 1919, Lancefield 1928b) were supported by studies in fresh human blood (Hare 1928, 1932) and in indirect bactericidal tests (Wannamaker et al. 1953). These factors have also been demonstrated in group G streptococci (Lawal et al. 1982). There are also other extracellular products that are leucocidal. These are streptolysin O and streptolysin S (Alouf 1980). Streptokinase has been suggested to favour the spread of streptococci by preventing the formation of fibrin clots around infectious lesions (Castellino 1979), but their role in pathogenicity is uncertain, except that there is now some evidence of its involvement in acute glomerulonephritis (Johnston and Zabriskie 1986). Other factors that damage tissue cells may contribute to the formation of the local lesion; these include cellular products such as mucopeptide and group polysaccharide (Cromartie et al. 1979).

1.3. THE LANCEFIELD GROUP C STREPTOCOCCI

Group C streptococci, like other members of the genus Streptococcus, are spherical, gram-positive cocci that divide in one plane, resulting in pairs and chains. These microorganisms are aerobic, facultatively anaerobic, capnophilic and catalase negative. Members of group C are distinguished from streptococci of other groups by the precipitin reaction described by Lancefield (1933); the antigenic determinant of the group-specific

carbohydrate is the rhamnose-N-acetylgalactosamine polysaccharide located on the cell wall (Wagner and Wagner 1975). The group C streptococci comprise the four species, S.equisimilis, S.dysgalactiae, S.equi and S.zooepidemicus that can be identified by physiological tests. Strains of S.milleri which cross-react with the Lancefield group C polysaccharide antigen may also be distinguished biochemically from the four species of Lancefield group C (Table 1).

S.equisimilis is by far the most commonly isolated group C streptococcus from humans (Stamm and Cobbs 1980) but it has also been isolated from infections in animals (Wilson and Salt 1978). S.dysgalactiae is an important cause of mastitis in cattle (Higgs et al. 1980). S.equi, the etiological agent of 'strangles' in horses, is the only biotype which seems host specific (Bryans and Moore 1972 , Bannister et al. 1985). Finally, S.zooepidemicus is a frequent agent of serious epidemic disease in animals (Wilson and Salt 1978) and occasionally in humans (Barnham et al. 1983,1987).

1.3.1. BIOCHEMICAL IDENTIFICATION AND GENERAL CHARACTERISTICS

The four species of group C streptococci may be identified by their biochemical properties, their reactions on blood agar, their host specificity and their pathogenicity for humans (Table 2). S.equisimilis was named by Frost and Engelbrecht (1940) and was initially classified along with the other three species on the basis of their fermentative properties. Simmons and Keogh (1940) showed a correlation between serological type and physiological

Table 2

Haemolysis, fermentation patterns and pathogenicity for animals and humans of Lancefield group C streptococci

Species of Streptococci	Haemolysis on Blood agar	Ability to ferment			Pathogenicity for	
		Trehalose	Sorbitol	Lactose	Animals	Humans
S.equisimilis	β	+	-	v	Uncommon	Common
S.zooepidemicus	β	-	+	v	Common	*Rare
S.equi	β	-	-	-	Common (horses)	Rare
S.dysgalactiae	α	+	v	v	Common (cows)	Rare

+: Ferments consistently
-: Does not ferment
v: Variable ability to ferment

* Increasing awareness of infections due to S.zooepidemicus.

characteristics of these organisms. They subdivided the human strains of group C (S.equisimilis) into seven sub-groups by their actions on aesculin, amygdalin, raffinose and lactose. Sherman (1937) distinguished between the human and animal isolates of group C using the three carbohydrates trehalose, sorbitol and lactose. Animal isolates fermented sorbitol and lactose but not trehalose. The 'human C' strains however did ferment trehalose but not sorbitol and were variable in their fermentation of lactose. From these findings it may be inferred that animal and human isolates of S.equisimilis are perhaps representatives of different species.

Synthetic substrates have often been used by histochemists for the detection and location of enzymes (Gomori 1957) and were later applied by microbiologists for use in identification tests (Humble et al. 1977). Twenty tests were incorporated in the API-20 Strep gallery (API Laboratories, France). This kit consists of a gallery of microtubes containing dehydrated substrates for the demonstration of enzymatic activity and the fermentation of sugars. The test kit could distinguish the four established species of Lancefield group C from other streptococci but was not satisfactory for distinguishing between strains of S.dysgalactiae and S.equisimilis (Colman and Ball 1984).

S.dysgalactiae was first described by Diernhofer in 1932 for strains causing mastitis in cattle. S.dysgalactiae does not yield a soluble haemolysin and produces either greening or no haemolysis at all on blood agar. It ferments trehalose consistently, but

fermentation of sorbitol and lactose is variable (Deibel and Seeley 1974). This organism may also be distinguished from the other species of Lancefield group C by the RK test (Skorkovsky 1973). A positive RK test is indicated by the growth of all S.dysgalactiae strains on a blood agar medium (3% nutrient agar, pH 7.2 with 5% defibrinated sheep blood) containing KCNS (2.25%).

S.equi was described as early as 1888 by Sand and Jensen as the causative organism of strangles, which is an acute infectious respiratory disease of horses. These organisms produce a soluble haemolysin but not a fibrinolysin. They do not ferment trehalose, sorbitol or lactose (Edwards 1932, Sherman 1937, Bannister et al. 1985).

Streptococcus zooepidemicus was named by Frost and Engelbrecht (1940). It produces a soluble haemolysin that is not related to either streptolysin O or S, and it does not produce streptokinase (Deibel and Seeley 1974). It ferments sorbitol but not trehalose (Ogura 1929, Edwards 1932). Ogura (1929) and Edwards (1932, 1933) recovered this organism only from animals and Sherman (1937) named it the 'animal pyogenes'. This species may be further identified by the identification of cell wall proteins (Moore and Bryans 1969). These protein antigens are type-specific for S.zooepidemicus. Moore and Bryans (1969) defined eight serotypes on this basis, all associated with equine diseases. These strains are no longer available (Pers.comm.M.Barnham).

1.3.2. EXTRACELLULAR ENZYMES OF S.EQUISIMILIS

S.equisimilis is the only species in group C that produces both of the extracellular enzymes streptokinase and streptolysin O (Kirby and Rantz 1943, Diebel and Seeley 1974). The streptokinase of S.equisimilis is antigenically distinct from the two streptokinases produced by the Lancefield group A streptococci (Dillon and Wannamaker 1965), and this extracellular product may contribute to the pathogenicity of these organisms. Recent work has indicated the usefulness of the group C streptokinase in the digestion of fibrin clots and has been applied clinically on a trial basis in the field of cardiology (Pers.comm.L.Jeffries).

Experiments have shown that the mortality in mice was greater (Krasner and Young 1959) and skin lesions in rabbits were larger (Krasner and Jannach 1963) in animals infected with streptokinase-producing streptococci than in those infected with streptococci that did not produce streptokinase. Streptokinase forms a complex with plasminogen activator; this complex catalyses the conversion of plasminogen to plasmin. Plasmin can cleave the third component of complement C'3, a chemotactic factor for polymorphonuclear leukocytes (Ward 1967). Plasmin can also stimulate the digestion of fibrin. Although the role of streptokinase in the pathogenicity of streptococci is unclear, it is possible that these substances contribute to the invasiveness of the organisms by preventing the formation of fibrin clots around infectious lesions (Castellino 1979).

The relationship of streptokinase to acute glomerulonephritis (AGN) has been indicated. A nephritis strain-associated protein (NSAP) was secreted by streptococcal strains from cases of AGN. When purified NSAP was subjected to amino acid analysis, it became apparent that this protein shared an amino acid composition profile with streptokinases isolated from groups A, C and G streptococci. Biochemical analysis confirmed that NSAP could act as a plasminogen activator, however, NSAP and streptokinase alone did not cleave the plasmin substrate. Therefore, there may be an association between streptokinase and NSAP in the pathogenesis of AGN unique to groups A, C and G streptococci (Johnston and Zabriskie 1986). Kiefer and Halbert (1976) demonstrated that human sera contained antibodies to the group C streptokinase.

Hyaluronic acid which is the component material of the capsule in some strains of group C streptococci is a polysaccharide composed of D-glucuronic acid and N-acetylglucosamine. It impedes the phagocytosis of these organisms and increases their virulence (Seastone 1939, Kass and Seastone 1944). Hyaluronidase has been referred to as 'spreading factor'. Alternatively, by breaking down the hyaluronic acid capsule, this enzyme may reduce the virulence of the organisms (Seastone 1943). Three streptococcal species can also possibly be distinguished by the relative amounts of hyaluronidase formed. S.equisimilis strains produce 5 to 10 times more hyaluronidase than those of S.zooepidemicus. Strains of S.equi did not show enzyme production (Balke et al. 1985). There are other extracellular enzymes produced by the group C

streptococci which include the deoxyribonucleases and nicotinamide adenine di-nucleotide glycohydrolase but the hyaluronidase appears the most useful as a diagnostic tool. The measurement of antibody to the group C hyaluronidase in patients with suspected group C infections has been used to confirm the diagnosis (Hallas and Widdowson 1982).

An enzyme-linked immunoassay (ELISA) was established for the assay of serum antibodies to the group C and G streptococcal group-specific carbohydrates. The specificity of this assay confirmed the usefulness of the test in providing evidence of infections by these organisms (Ayoub et al. 1986).

1.3.3. GROUP C STREPTOCOCCAL DISEASE IN MAN AND ANIMALS

Group C streptococci are a common cause of infection in animals and comprise the species S.equi, S.equisimilis, S.dysgalactiae and S.zooepidemicus. Animal diseases include mastitis in cows, lymphadenitis in guinea pigs (Duma et al. 1969, Olson et al. 1976), jaw abscesses in swine (Olson et al. 1976) and equine strangles.

Group C streptococci, notably the biotype S.equisimilis, can be the cause of serious infections in man, which include bacteraemia (Rosenthal and Stone 1940, Sanders 1963, Lawrence and Cobbs 1972, Finnegan et al. 1974, Mohr et al. 1979), pneumonia (Mohr et al. 1979) and endocarditis (Feinberg and Shabino 1985). S.equisimilis is the cause of most cases of human disease due to group C streptococci and was labelled the 'human C' streptococcus by

Sherman (1937).

Asymptomatic pharyngeal carriage of group C streptococci in humans has been recognised. Hare (1940) combined the data from his own and six other studies of group C streptococcal infections and calculated that approximately 3% of some 3000 subjects were carriers. Sore throat and tonsillitis due to group C streptococci were first reported by Griffith in 1934: his types 7, 20 and 21 streptococci were later identified as S.equisimilis. In a review of group C streptococcal infections, Hutchinson (1946) described thirty three patients with tonsillitis, six of whom had septic throats with fever, enlarged cervical lymph nodes and necrotic debris on the tonsils. An epidemic of pharyngitis at a school for children with learning disabilities was reported by Benjamin and Periello (1976).

S.dysgalactiae causes mastitis in cattle and may be isolated from their milk (Gillespie and Timoney 1981). Only once has what was possibly S.dysgalactiae been identified as a human pathogen (Quinn et al. 1978).

Strangles is an acute respiratory disease of horses that is caused by S.equi. Only young animals not previously infected by S.equi seem to be susceptible. Transmission occurs by inhalation or ingestion of droplets of nasal discharge. Preventative measures include the isolation of infected animals, prophylactic administration of benzylpenicillin G and immunisation of uninfected

animals (Bryans and Moore 1972). The only reported case of human disease due to this organism occurred in a middle aged woman with bacteraemia (Duma et al. 1969).

S.zooepidemicus is a common cause of serious, epidemic disease in domestic animals. This biotype has been responsible for septicaemia in horses, cattle, pigs, sheep, foxes and guinea pigs, for pneumonia in horses, abortion in horses and pigs and abscesses in horses (Edwards 1933, Blood and Henderson 1963, Rumbaugh et al. 1978). Recent reports have described this species as a cause of disease in humans. Duca et al. (1969) and Barnham et al. (1983) both reported separate incidents in which pharyngitis followed by nephritis was caused by consumption of unpasteurised milk containing S.zooepidemicus. An earlier cluster of three patients with severe infection described by Ghoneim and Cooke (1980) was later shown to be due to contamination of unpasteurised milk from a common source. Frost and Engelbrecht (1940) isolated this organism from the throats of healthy dairy employees and also from patients with sore throats in direct contact with, or consuming milk from infected cows.

S.zooepidemicus was also implicated epidemiologically in a unique outbreak in the USA in which the vehicle of infection was soft cheese prepared from unpasteurised cows milk (Anon.1983). The cause of this outbreak was contaminated milk from cows with udder infections due to S.zooepidemicus. Because few laboratories determine routinely the species of group C streptococci, the number

of human infections caused by S.zooepidemicus is not known. The few reports available suggest that it is an unusual and incidental infection in man, probably acquired directly or indirectly from animals. It seems therefore that it should be regarded as a zoonosis and included in the list of infections that may be transmitted by milk (Galbraith et al. 1982).

Each of the four species of group C streptococci has a unique spectrum of pathogenicity for animals and humans but the virulence factors are unknown. There are several serotypes within certain species : at least eight antigenically distinct types of S.zooepidemicus (Moore and Bryans 1969) and more than ten distinct types of S.equisimilis will be described later in this thesis.

1.3.4. ANTIBIOTIC RESISTANCE AND TREATMENT OF DISEASE

The antibiotic of choice for the treatment of group C streptococcal infections is benzylpenicillin. For patients allergic to penicillins and cephalosporins, erythromycin or even clindamycin and chloramphenicol have been used as substitutes. The organisms are invariably inhibited by 0.1 μg/ml of penicillin (Mohr et al. 1979). For the treatment of specific diseases such as endocarditis, large doses of benzylpenicillin are recommended (Mohr et al. 1979). Portnoy et al. (1981) however, demonstrated pencillin tolerance in group C streptococci. They found it to be a common occurrence. Sixteen of the seventeen strains tested showed penicillin tolerance with a 32-fold or greater difference between the minimal inhibitory concentration (MIC) and the minimal

bactericidal concentration (MBC). The isolates were from many different sites. This tolerance to penicillin may explain the poor outcome of some serious group C streptococcal infections, for example, endocarditis treated with penicillin alone (Anderson and Cruickshank 1982). Portnoy and his colleagues recommended that for serious infections, the minimum bactericidal concentration for penicillin be determined and that for initial therapy, the use of gentamicin in addition to penicillin be considered pending results of susceptibility tests.

For the animal infections caused by group C streptococci, vaccines have been developed and are now commercially available for use in horses for the prevention of infections due to S.equi (Woolcock 1975, Srivastava and Barnum 1981).

1.4. THE LANCEFIELD GROUP G STREPTOCOCCI

Group G streptococci were first described by Lancefield and Hare in 1935. Their strains were isolated originally from parturient women. Although not as prevalent as the group A streptococci, group G have been identified as the aetiological agent in several different infections.

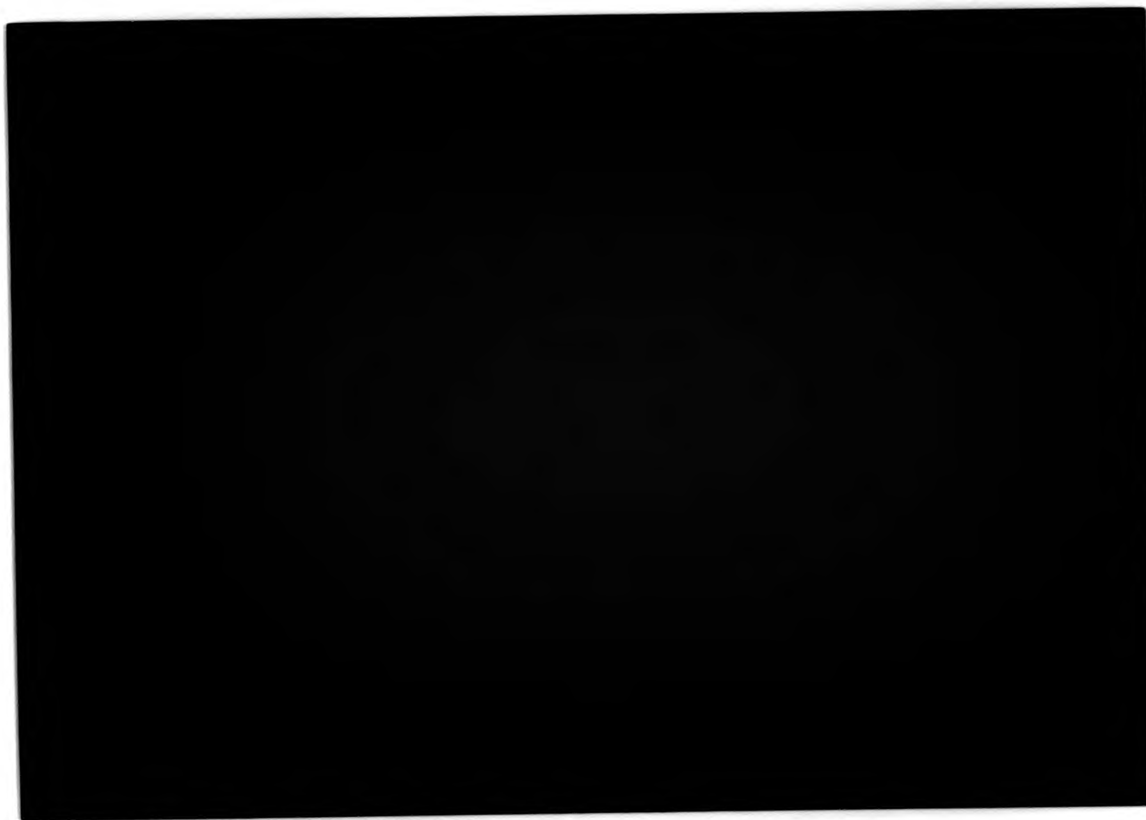
1.4.1. GENERAL CHARACTERISTICS

Group G streptococci are similar culturally to the pyogenic streptococci of Lancefield groups A and C. Most are β-haemolytic on blood agar due to the production of a soluble haemolysin,

streptolysin S. Griffith's (1934) type 16T protein strains also possessed the group G polysaccharide antigen (Wilson and Miles 1946). Because of their similarity to S.pyogenes, Smith and Sherman (1938) referred to them as 'pyogenes-like' and Simmons and Keogh (1940) described them as the 'large-colony type'. Two different colony forms of group G streptococci have been recognised on blood agar, the 'minute' and the 'large' colony form (Smith and Sherman 1938). The 'minute' strains with broad zones of haemolysis (Fig.1) were named by them Streptococcus anginosus. The characteristic large, 'matt' colony form (Fig.1) currently remains nameless in Bergey's Manual of Determinative Bacteriology. It has been referred to as S.canis, because of its association with canine infections (Stafseth et al. 1937). Devriese et al. (1986) have proposed that all animal 'large-colony' group G streptococci be designated S.canis. They have shown that strains of S.canis differ from the human isolates of group G by DNA hybridisation tests and by their possession of a rare cell wall peptidoglycan type (namely lysine-threonine-glycine) that is common to all animal isolates. Thus, the human isolate remains to be named.

Simmons and Keogh (1940) analysed seventy eight group G streptococci of which 8 were of the minute colony form. At least 3 serological types of the large colony strains were identified by slide agglutination techniques in their study. Only one type was recognised among the minute strains. Simmons and Keogh's strains have been discarded.

Fig.1 'Large' and 'minute' colony forms of Lancefield group G streptococci



'Large' colony (x4)

Streptococcus sp.

S.canis: animal sp.

'Minute' colony (x4)

S.milleri

1.4.2. BIOCHEMICAL IDENTIFICATION

Although serological grouping is the method of choice for identification of pyogenic streptococci, there are several biochemical tests which can be used to identify presumptively the beta-haemolytic streptococci. In particular, the fermentation of ribose and the production of pyrrolydonylarylamidase (PYRA) have been found useful (Colman and Ball 1984). Pyogenic streptococci of human origin that belong to the Lancefield groups C or G ferment ribose but do not hydrolyse PYRA whereas S.pyogenes (Lancefield group A) gives the reverse reactions. Simmons and Keogh (1940) subdivided the 'minute' and 'large' colony forms on the basis of their fermentation patterns of four carbohydrates. These were starch, glycerol, raffinose and amygdalin. The 'large' colony varieties fermented starch and glycerol and the 'minute' forms gave positive reactions with raffinose and amygdalin.

Identification of beta-haemolytic streptococci from animal sources has traditionally relied heavily on carbohydrate fermentation patterns, with special emphasis on the substrates lactose, trehalose and sorbitol (Evans 1944). When Stafseth and his colleagues (1937) applied a similar approach to canine strains, some of which were subjected to serological grouping and turned out to be also group C, a fourth fermentation pattern was added to the three recognised earlier based on the fermentation of lactose, trehalose and sorbitol. The name S.canis was proposed for organisms conforming to this pattern (lactose positive, and with no action on trehalose and sorbitol). Biberstein and co-workers

(1980) confirmed the findings of Stafseth and identified canine isolates of Lancefield group G by their ability to ferment lactose only; but not sorbitol or trehalose.

1.4.3. EXTRACELLULAR PRODUCTS OF LANCEFIELD GROUP G

Group G streptococci produce several extracellular enzymes similar to those produced by S.equisimilis (Lancefield group C). These include streptolysin O, streptolysin S (Alouf 1980) and hyaluronidases (Hallas and Widdowson 1982). Nuclease production among group G streptococci is relatively rare (Ferrieri 1980).

1.4.4. GROUP G STREPTOCOCCAL DISEASE IN MAN AND ANIMALS

Group G streptococci have been isolated from a variety of human and animal infections. These are being reported regularly (Anon 1984, Vartian et al. 1985). Infections in premature babies are amongst one of the many recently documented (Baker 1974, Ancona et al. 1979, Appelbaum et al. 1980, Dyson and Read 1981). Since streptococci of group G, like those of group B, can be carried asymptomatically in the female genital tract neonatal infection is not unexpected (Lancefield and Hare 1935). The presence of maternal or neonatal predisposing factors, such as premature rupture of the amniotic membrane, prematurity, prolonged or difficult labour, increases the likelihood of infection (Baker 1974, Ancona et al. 1979). Maternal postpartum septicaemia due to group G streptococci has also been described (Colebrook and Purdie 1937, Hill and Butler 1940, Ramsay and Gillespie 1941, Filkir and Monif 1979).

Group G streptococci exist as part of the normal flora of the throat, or skin, and occasionally of the intestinal tract (Duma et al. 1969). Most infections occur either in the skin or in wounds (Feingold et al. 1966, Duma et al. 1969, Armstrong et al. 1970). Septicaemia is not uncommon and may be associated with endocarditis (Duma et al. 1969, Parker and Ball 1976, Bouza et al. 1978, Auckenthaler et al. 1983). Their presence in the upper respiratory tract may be associated with sore throat and swollen lymph glands (Feingold et al. 1966, Hill et al. 1969). Cases of septic arthritis due to group G are not as rare as was previously thought and there have been several recent case reports (Bradlow et al. 1982, Coto et al. 1982, Fujita et al. 1982, Lin et al. 1982, Nakata et al. 1983, Gaunt and Seal 1986).

Group G streptococci have also been thought to cause occasional epidemics of sore throat (Hill et al. 1969). One epidemic was foodborne (Stryker et al. 1982), another outbreak of pharyngitis occurred at a college (McCue 1982). These outbreaks demonstrated that group G streptococcal sore throat can be clinically indistinguishable from that caused by the group A streptococci and it results in rises of the anti-streptolysin O (ASO) titre (McCue 1982).

In animal populations, group G streptococci have long been recognised as significant causes of acute infections especially in dogs (Biberstein et al. 1980), cats (Tillman et al. 1982) and

cattle (Bergner-Rabinowitz _et al_. 1981).

1.4.5. ANTIBIOTIC RESISTANCE AND TREATMENT OF DISEASE

Group G streptococci are susceptible _in vitro_ to various antimicrobial agents, including benzylpenicillin, most cephalosporins, vancomycin and erythromycin. Lam and Bayer (1983) determined the bactericidal activity of ten clinically useful antibiotics and benzylpenicillin, ampicillin and cefotaxime were the most active. All group G strains tested were killed by 0.08 µg/ml or less of these three agents. None was found to be tolerant to the killing action of these agents although Finch and Aveline (1984) did find tolerance in several of their strains to erythromycin, vancomycin or penicillin. Rolston _et al_. (1984) suggested that tolerance did occur frequently enough in these organisms to justify careful susceptibility testing including the determination of MBCs. The conclusion of Vartian _et al_. (1985) stated that a combination of a cell-wall antibiotic with an aminoglycoside should be considered in the treatment of serious systemic group G streptococcal disease, especially in immunosuppressive diseases, although the clinical advantage of such combinations has not been proven.

1.5. ANTIGENIC CONSTITUTION OF GROUP C AND GROUP G STREPTOCOCCI

The chemical content of the cell walls of the Lancefield group C and group G streptococci are similar in many ways to those of

Lancefield group A (Lancefield 1942, Schmidt 1952, Curtis and Krause 1964a). The major components are mucopeptide, the group specific carbohydrate and the cell wall protein antigens (Salton 1952, Barkulis and Jones 1957). Lipotechoic acid is present in strains of group A but work has not been extended to detect its presence in group C and group G streptococci.

1.5.1. THE CELL WALL - structure and composition

Rhamnose has been identified as the main component in the carbohydrate of several groups of streptococci, including groups A, C and G (McCarty 1952, Schmidt 1952, Kilpper-Bälz and Schleifer 1984). The trypsinised cell walls of groups A, C and G streptococci contain two major components, the mucopeptide matrix consisting of N-acetylmuramic acid, N-acetylglucosamine and four main amino acids which are alanine, glutamic acid, lysine and glycine (Krause and McCarty 1962). Kilpper-Bälz and Schleifer (1984) found rhamnose was the major characteristic component of the cell wall polysaccharide and various neutral sugars (glucose, galactose) and amino sugars (glucosamine, galactosamine) could be detected as additional constituents of the cell-wall polysaccharide. Thus, the chemical composition of the streptococcal cell wall is relatively well known, and one suggestion is that it is arranged in three concentric layers, composed from within outwards, peptidoglycan or mucopeptide, polysaccharide and protein (Krause 1972). Electron micrograph studies have shown clearly an ultrastructural image of the cell wall from these organisms (Wagner et al. 1978, 1979). The studies

demonstrated an electron-dense layer (perhaps the cytoplasmic portion of the cell) a layer of medium electron density (peptidoglycan) and a further thin electron-dense layer often bearing filamentous protrusions (protein). Removal of these protrusions by trypsin suggested their protein nature (Swanson et al. 1969).

The chemical composition of the peptidoglycan

The appearance in electron micrographs of the streptococcal cell wall and the fact that proteins can easily be removed by proteolytic enzymes without affecting the viability of the streptococci (Lancefield 1943) led to the belief that the components of the wall are arranged in concentric layers (Krause 1972). The architecture of the streptococcal cell wall may however represent a complicated mosaic structure in which peptidoglycan forms a skeleton traversing the wall as a network (Stollerman 1975, Rýc et al. 1979). Streptococcal peptidoglycan, the residue remaining after hot formamide extraction, is composed of N-acetylglucosamine, N-acetylmuramic acid, alanine, glutamic acid, lysine and small variable amounts of glycine (Karakawa and Krause 1966).

Schleifer and Kandler (1972) concluded that the peptidoglycan or murein structure was a valuable taxonomic marker amongst the Gram positive organisms. Their studies on the amino acid composition and amino acid sequence of streptococcal peptidoglycans revealed 12 different 'murein' types. They concluded that a closely related

cluster may be formed by S.pyogenes (group A), S.equisimilis (group C) and Streptococcus sp. (group G), the predominant amino acids being lysine and alanine. On the basis of cell wall composition and nucleic acid hybridisation, the pyogenic streptococci were divided into five clusters, the finest cluster consisting of groups A, C, G and L (Kilpper-Bälz and Schleifer 1984).

The capsule

The capsule is produced during the early stages of growth (Morison 1940, McClean 1941, Pike 1948a and b), and may be removed during the stationary phase if the extracellular enzyme hyaluronidase is produced by the cell. The presence of hyaluronic acid in the growth medium will stimulate the production of hyaluronidase, (McClean 1941). Many human strains of group C and group G produce hyaluronidase (Hallas and Widdowson 1982). There is little information available on the capsules of group C and Group G streptococci with the exception of capsulated animal strains of group C, notably S.equi (Prescott et al. 1982).

The group polysaccharide antigen

This is a group polysaccharide which determines the serological specificity (Lancefield 1933, Krause and McCarty 1962). In group A streptococci the antigenically dominant sugar is N-acetylglucosamine. In Lancefield group G an additional sugar, galactose, is present in the cell wall carbohydrate and it is not present in the walls of the other groups. The group G antigen is composed of L-rhamnose, galactosamine and galactose. L-rhamnose

is the major antigenic determinant (Curtis and Krause 1964a). Streptococcal groups A and C also contain L-rhamnose, but the serological activity of the rhamnose side chains is masked by the terminal amino sugars N-acetylglucosamine and N-acetylgalactosamine respectively (Curtis and Krause 1964b). Both groups C and G contain rhamnose and galactosamine, as part of the polysaccharide matrix, but galactosamine is present in larger quantities in group C (35.1%) than in the group G (17-20.6%) carbohydrate, (Curtis and Krause 1964a). It appears therefore that the major determinant of antigenic specificity in group G is rhamnose, and in group C the N-acetylgalactosamine residues (Krause 1963).

The group polysaccharide may be extracted from whole cells with dilute hydrochloric acid at 100°C (Lancefield 1928b, 1933), with formamide at 170°C (Fuller 1938), by the autoclave procedure of Rantz and Randall (1955) and among other methods the nitrous acid extraction procedure of El Kholy et al. (1974), and also by the digestion of the cell walls by muralytic enzymes released by Streptomyces albus (Maxted 1948). It is also resistant to digestion by proteolytic enzymes.

T protein

The T-protein antigen apparently plays no part in pathogenicity but is useful in the serological and epidemiological identification of particular strains (Griffith 1934). It is trypsin-resistant on the living cell but can be removed intact from heat-killed cells by this enzyme (Pakula 1951). The T proteins are slowly destroyed by

pepsin and are generally sensitive to acid, but have variable sensitivity to heat at pH 7.0 (Pakula 1951). Griffith's (1934) original classification of pyogenic streptococci into different T-types included types 7, 16, 20 and 21 which were later found not to belong to Lancefield group A but to be strains of either group C or group G (Hare 1935).

M protein

M protein is an antiphagocytic molecule and is known to be responsible for one form of type specificity (Lancefield 1928b). Streptococcal M proteins have been described in Lancefield groups A, C and G (Griffith 1934, Maxted and Potter 1967, Lawal et al. 1982). The M protein is thought to inhibit phagocytosis (Wiley and Wilson 1956) and streptococci bearing it are therefore able to survive in human blood. Humans respond to acute streptococcal infections by forming type-specific opsonic antibody to the M protein (Rothbard 1945) which can be measured in vitro using the bactericidal test (Lancefield 1957). Although the human response to the group A streptococcus has been studied extensively, epidemiological evidence of the protective role of M-type-specific antibody to group A is scanty. Giuirguis et al. (1982) studied the spread of certain group A serotypes amongst 64 families in Egypt. They showed that type specific serum bactericidal antibody does not protect against throat carriage of the same organisms and thus type-specific immunity if it exists must perhaps be mediated in another way such as by local antibody or through protection of infection.

Swanson et al. (1969) originally showed by a ferritin-labelled antibody technique that the M protein exists as 'fimbriae-like' structures on the cell surface. This was later confirmed by Ryc et al. (1979). The structural features responsible for the common antiphagocytic function are not well understood due to the lack of information regarding the amino acid sequence of these proteins. Manjula and Fischetti (1982) demonstrated that the streptococcal M protein of Lancefield group A is composed of a flexible coiled -coiled fibrillar structure able to extend more than 600A from the surface of the organism. Electron micrograph studies of the cell surface later revealed that the cell wall of the group A streptococcus is not as rigid as was originally thought (Fischetti and Fazio-Zanakis 1985).

The cell surface of the Lancefield group C and group G streptococci has not been fully described. Biochemical characterisation of the cellular components of these organisms has not yet been done.

The M proteins are acid and heat resistant, trypsin and pepsin sensitive. The M antigen is usually extracted from the cells at 100°C at pH 2.0 but may also be detected in the growth supernate.

Other protein antigens

There are several other protein antigens present in the group A streptococcal cell wall but these have not yet been fully defined within the group C or group G streptococci. These are the R, B

and 'T-like' antigens which apparently share some of the characteristics of M proteins, in that they are resistant to heat and acid. They do not impede phagocytosis and their biological significance is unknown (Rosendal 1950, Lancefield and Perlmann 1952, Maxted 1953, Hambly 1958).

M-associated protein

This cell wall protein is not type-specific as is the M protein but is closely associated with the latter (Widdowson et al. 1971). M-associated protein (MAP) can be detected in the acid extracts of all types of M-positive streptococci, but not M-negative variants, by means of a complement fixation test with the serum of a human donor who has antibody to MAP. The antigen is also present in the acid extracts of certain streptococci of groups C and G where there is evidence for the presence of an M or M-like antigen (Lawal 1982).

MAP cannot be separated from type-specific M antigen by protein purification methods. Also, purification of M protein always results in a parallel increase in activity of MAP and the type determinant, suggesting that the two may form part of the same molecule or complex. Separation has been achieved by treatment of cell bound M antigen with streptococcal proteinase which releases MAP (Widdowson et al. 1971).

1.5.2. THE PROTOPLAST MEMBRANE

The membrane of Lancefield group A is very similar to that of group

C and group G. It is known to possess a group-specific antigen which cannot be found in the cell wall or cytoplasm and which shares some determinants with membrane antigens of group C and group G streptococci. In the group A streptococci it is a semi-permeable lipoprotein membrane containing lecithin, glycolipids and straight-chain fatty acids (Freimer et al. 1959, Freimer 1963). It is composed also of a wide variety of amino acids with glucose and traces of rhamnose and hexosamine.

1.5.3. DNA BASE COMPOSITION

DNA base compositions are widely used as reference tests in taxonomy. Weissman et al. (1966) used a DNA-RNA hybridisation technique to measure the relationships among Lancefield groups and serotypes of streptococci. The results indicated that the Lancefield groups examined could be differentiated by this method. The data provided evidence which suggested that Lancefield groups A, C, F and G have a closer relationship to each other than group D.

The DNA base composition of streptococci is between 34-46% (GC ratios). The study of Farrow and Collins (1984) indicated that *S.dysgalactiae*, *S.equisimilis* and streptococci of Lancefield groups C, G and L could be regarded as a single species, based on DNA base composition, DNA-DNA hybridisation and biochemical tests. Strains of *S.equi* and *S.zooepidemicus* were found to be closely related on the basis of DNA-DNA hybridisation. *S.dysgalactiae* strains were found to possess a mol % G + C range of 38.5 to 39.8, which was in

accord with data published by Garvie et al. (1983). S.equisimilis and streptococci of serological groups G (large colony type) and L possessed a similar mol % G + C range of 38.1 to 40.2, but strains designated S.zooepidemicus had higher values of 41.3 to 42.7 mol % G + C.

The studies of Kilpper-Bälz and Schleifer (1984) indicated that the classical pyogenic streptococci could be divided into five homology clusters. Based on these studies they suggested the term pyogenic streptococci be confined to the first cluster which consisted of serological groups A, C, G and L. They also indicated the close relationship among groups G (large-colony), L and S.dysgalactiae, on the one hand, and S.equi, S.zooepidemicus on the other. Therefore, on the basis of DNA hybridisation tests it might be argued that groups A, C, G and L form a natural 'grouping'.

1.5.4. SEROLOGICAL CLASSIFICATION OF LANCEFIELD GROUP C AND GROUP G

The most useful single method for the identification of these streptococci is serological grouping. In 1933 Lancefield first grouped streptococci according to their cell wall carbohydrate, using hydrochloric acid extraction and the capillary precipitin ring test. The technique is widely used today with little modification if any. Other chemical extraction methods include the formamide procedure of Fuller (1938), the autoclave procedure of Rantz and Randall (1955) and the nitrous acid extraction method of El Kholy et al. (1974). All these require heat and a 24h pure culture of the streptococci. Other methods include the use of

lytic enzymes notably the Streptomyces albus enzyme technique of Maxted (1948). The procedures do not require a pure culture and the processing time is much shorter. The growth may be taken from a primary plate, extracted for 30-60 min, centrifuged and tested. The carbohydrate antigens may then be identified by the classic antigen-antibody precipitation reaction using either the ring test, or radial gel diffusion techniques. In the capillary ring test, a precipitate ring forms at the interface of the extracted antigen and group specific antiserum (Lancefield 1938). With the gel technique, the diffusion in agar occurs radially from a well of antiserum in the centre which is surrounded by wells containing extracted antigen (Ouchterlony 1958). There are several newer, more rapid techniques available for streptococcal grouping which include countercurrent immunoelectrophoresis (CIE), (El Kholy et al. 1974), the fluorescent antibody method (Moody et al. 1958) and the slide agglutination techniques (Christensen et al. 1973, Lue et al. 1978, Efstratiou and Maxted 1979).

CIE is a gel diffusion technique which adds an electric current to expedite the antigen-antibody reaction (Avrilommi et al. 1978). This is in fact a more sensitive technique than the capillary or gel diffusion precipitin tests and further absorption of antisera is required to prevent any cross-reactions. The fluorescent antibody method of Moody et al. (1958) uses direct smears from two-hour broth cultures or 24-hour agar cultures. The reagent is a group specific globulin labelled with fluorescein-isothiocyanate. The stained streptococci appear as apple-green fluorescent coloured

cells under a fluorescent microscope.

The slide co-agglutination techniques are now used widely. The original reagent consisted of formaldehyde heat treated staphylococci coated with group specific antibody. Staphylococcal protein A is a unique cell-wall associated protein showing a high affinity for the Fc portion of immunoglobulins G-1, G-2 and G-4 of humans. The binding orients the Fab portion outward for reaction with the streptococcal antigen. The antigen may be extracted using enzyme digestion, by the use of proteolytic enzymes such as pronase. There have been several modifications of this technique.

In 1978 Lue et al. adopted the technique of coating latex particles with the group specific antisera, instead of the staphylococcal Protein A. The antigen is extracted enzymatically for 10-30 minutes at 37°C and tested against the sensitised latex particles using the slide agglutination techniques. These agglutination methods are very sensitive and antisera must be fully absorbed to reduce the incidence of cross-reactivity.

1.5.5. TYPING OF LANCEFIELD GROUP C AND GROUP G STREPTOCOCCI

The epidemiology of both these Lancefield groups has been poorly understood because of lack of a well-defined typing scheme. Numerous typing methods have been described for both these groups but are not widely used.

The most useful typing schemes are those applicable to the

cell-wall antigens, notably the protein antigens as is well documented in Lancefield group A. The techniques of the respective typing systems for the Lancefield group A streptococci are determined by the properties of these antigens. T-protein antigens are destroyed by hot acid treatment but resist trypsinisation and M antigens may be extracted with 0.2N HCl at 100°C and are trypsin sensitive. The main objective of typing these organisms in epidemiological studies is to identify the M protein antigen if anti-M sera are available for that particular type. However, nearly all strains may be characterised by their T-antigen pattern. T antigens are usually identified by the agglutination of trypsinised suspensions of the streptococci (Griffith 1934). The M protein is usually detected by testing the extracted antigen by means of precipitation in double gel diffusion (Rotta et al. 1971). These protein antigens have also been described in Lancefield group C and group G streptococci (Griffith 1934, Maxted and Potter 1967, Lawal 1982). But, typing schemes for these organisms based upon their cell wall antigens have not yet been established.

Bacteriophage typing systems have been reported (Vereanu and Mihalcu 1979). A set of 16 temperate phages was developed and the lysotyping scheme identified 71% of S.equisimilis (Lancefield group C). This system classified the strains into seven groups and phage lysotypes. The phage preparations were only active on group C streptococci of human origin (Mihalcu et al. 1982). Phages have also been described in group G streptococci (Colón et al. 1971),

but a scheme has not yet been developed for these organisms. The phages isolated were not however host serogroup specific and could not be applied epidemiologically.

An inhibitor 'fingerprinting' technique was used by Schofield and Tagg (1983) to examine strains of Lancefield group C for the production of inhibitory activity. Their study indicated that 18% of 50 group C streptococci were inhibitor producers. They examined three of the species of group C (S.equisimilis, S.dysgalactiae, S.zooepidemicus) and all produced a degree of inhibitory activity. A typing scheme was not established. A similar system based on the production of inhibitory agents was also devised for group G streptococci (Tagg and Wong 1983). In their study 28 of 50 strains of human origin exhibited some degree of inhibitory activity. But, none of the 30 strains of animal origin demonstrated this at all.

Earlier schemes were mainly centred on the biochemical typing of these organisms as previously described (Simmons and Keogh 1940). However, recently biotyping and exoenzyme profiles for the differentiation of human and bovine isolates was described (Clark et al. 1984). They identified eight distinct biotypes within the species based on the fermentation of trehalose, lactose, raffinose and the hydrolysis of aesculin. In conjunction with the production of different exoenzymes (for example, DNase, protease, lipase, lecithinase, hyaluronidase, fibrinolysin and others) by these organisms they were able to differentiate between human and

bovine isolates of group G.

Restriction enzymes have also recently been applied to the identification of group C streptococci, (S.equisimilis and S.zooepidemicus) by the determination of HindIII DNA fingerprints for these organisms. This was applied to an outbreak of acute glomerulonephritis in Bucharest, Romania in conjunction with other schemes. This method was devised and applied by S.Skjold, University of Minneapolis, Minnesota, U.S.A.

To date, there has not been an established epidemiological typing scheme for the Lancefield group C and group G streptococci of human or animal origin.

OBJECTIVES OF THE INVESTIGATION

1. To establish whether, or not, haemolytic streptococci of Lancefield group C and group G are capable of causing outbreaks of infection in man.

2. To this end, study random isolates for biochemical or serological properties that could provide the basis for a typing scheme.

3. To apply such a scheme to strains isolated in clinical laboratories to see whether there is an association between any particular types and sites or forms of infection.

4. To examine strains from clusters of infection when the epidemiological evidence is consistent with spread of an aetiological agent and to determine whether the results of typing the strains are consistent with such data.

5. To provide basic information on the properties of the typing scheme.

2. MATERIALS AND METHODS

2.1. MEDIA

Blood broth

This was the medium used for the resuscitation of lyophilised cultures. Sterile defibrinated horse blood (5% v/v) was added to Nutrient Broth No. 2 (Oxoid) and was dispensed aseptically in 2ml volumes in 3 x 1/2in. cotton wool plugged tubes. The sterility of the medium was checked by incubation at 37°C for 48h. The broths were stored at 4°C. For longer storage of cultures, glycerol (16% v/v) was added to nutrient broth and sterilised by autoclaving at 115°C for 20 min. Horse blood (5% v/v) was added to the medium which was distributed in 1ml volumes and checked for sterility as above. The glycerol blood broth cultures were stored at -20°C.

Colindale-Todd Hewitt broth

One litre of water was added to 454g of fat-free minced beef and the mixture was stirred and left at 4°C overnight. It was subsequently heated to 80°C and kept at this temperature for 30 min. The infusion was then strained, clarified by filtration and the pH was adjusted to 7.0 with 10M NaOH after the addition of 20g peptone. To each litre of medium the following were added, $NaHCO_3$, 2g; glucose, 2g; NaCl, 2g and $NA_2HPO_4.12H_2O$, 1g. The broth was then autoclaved at 115°C for 10 min in 50ml volumes. The final pH of the medium was checked; this is usually 7.8 and does not require any further adjustment. The sterility of the medium was tested by incubating at 37°C for 18h.

Difco-Todd Hewitt broth with Neopeptone

30g Bacto-Todd Hewitt broth (Difco (UK) Ltd, West Molesey, Surrey) and 20g Neopeptone (Difco) were dissolved in 1 litre of distilled water. The medium was additionally buffered with 0.74g of $Na_2HPO_4.2H_2O$ and 0.13g of $NaH_2PO_4.2H_2O$ per litre. The pH was adjusted to 7.4 and the medium sterilised by autoclaving for 5 min at 115°C. The sterility was checked as above.

Oxoid-Todd Hewitt broth with Neopeptone

Todd Hewitt broth (Oxoid Ltd, Basingstoke, Hants.),(36.4g) was dissolved together with Neopeptone (Difco) (1% w/v in cold distilled water per litre) and the pH was adjusted to 7.6 - 7.7 with 10N NaOH (approx. 0.6 - 0.8ml per litre). The broth medium was then sterilised by autoclaving for 20 min at 115°C and then checked for sterility as above.

Glucose Nutrient Broth

Glucose (0.5% w/v) was added to Nutrient Broth (Oxoid No.2) and the pH was adjusted to 7.6 before sterilisation of the medium at 115°C. The sterility was tested as above.

Broth saline

Five ml of Difco-Todd Hewitt broth with Neopeptone was added aseptically to 100ml of sterile physiological saline. The sterility was tested as before.

Blood agar

Layered plates were prepared with peptone water agar as a base layer (Cruickshank et al. 1975). The top layer contained 5%(v/v) sterile defibrinated horse blood in Blood Agar Base Nutrient Agar (Oxoid CM 55) supplemented with yeast extract (0.3% w/v).

Columbia blood agar

Sterile defibrinated horse blood (Oxoid, 5% v/v) was added to Columbia agar (Oxoid, CM 331) and poured over a thin layer of peptone water agar.

2.2. BACTERIAL CULTURES

Selection

The streptococci examined in this study were isolated, in the main, from hospitals in the U.K., but some were referred from overseas. They were either random isolates from non-related sources and implicated in a disease or they were possible causes of hospital-acquired outbreaks. Stock cultures of group C and group G streptococci were also examined (Table 3).

Storage and culture

All the isolates were cultured onto blood agar either aerobically at 37°C or in the presence of air plus 5% CO_2 at 37°C. Subcultures from single colonies were used for all the experiments where possible. For storage all strains were either freeze-dried or subcultured into glycerol blood broth and stored at -20°C.

Table 3

Stock cultures of Lancefield group C and group G streptococci examined for the presence of protein antigens

Strain no.	Species	Lancefield group
NCTC 4540	S.equisimilis	C
NCTC 5370	"	"
NCTC 5371	"	"
NCTC 4669	S.dysgalactiae	"
NCTC 4335	"	"
NCTC 4671	"	"
NCTC 9682	S.equi	"
NCTC 6176	S.zooepidemicus	"
NCTC 5969	Streptococcus sp.	G
NCTC 7932	"	"
NCTC 9603	"	"
2832*	"	"
2440*	"	"

*Stock cultures isolated in the West Indies.

2.3. BIOCHEMICAL AND ENZYME IDENTIFICATION OF LANCEFIELD GROUP C AND GROUP G STREPTOCOCCI

Commercial identification kits were used to identify the streptococci for this study. Each strain was assigned to a species on the basis of results obtained with the API 20 STREP kit (API Laboratory Products Ltd., Basingstoke, Hants) supplemented with tests for sensitivity to bacitracin (0.1 unit disc) and serological grouping. The API ZYM kit was used to identify further the streptococci by their enzymatic activity on different substrates.

2.3.1. API 20 STREP

This kit is composed of a strip of 20 small cupules containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars. A dense suspension was prepared from a pure culture and was used to rehydrate the enzymatic substrates. The growth medium for the API test system was Columbia blood agar. The growth suspension was prepared from two overnight plate cultures of the organism to achieve turbidity between a McFarland No.5 and No.6 standard. The metabolic end products produced during the incubation period were either revealed through spontaneous colour reactions or by the addition of reagents. The fermentation tests were inoculated with an enriched medium which reconstituted the sugar substrates. Fermentation of carbohydrates was detected by a shift in the pH indicator. Tests

were incubated at 37°C and read at 4h and 24h (see Appendix 1).

Composition of media and reagents

The API 20 STREP medium contained cystine 0.5g, tryptone 20g, sodium chloride (NaCl) 5g, sodium sulphite 0.5g, phenol red 0.17g per litre in distilled water. The final pH was 7.8. The bacterial suspension medium was sterile distilled water. The colour reagents used were; 7% (w/v) ninhydrin in 2-methoxy-ethanol for the detection of free glycine in the hippurate test. Reagents for the detection of acetoin were; potassium hydroxide 40% (w/v) in distilled water and alpha-naphthol 6% (w/v) in ethyl acohol. Reagents for the detection of enzymatic activity were; Zym A; Tris-hydroxymethyl-amino-methane 250g, hydrochloric acid (37%) 110ml, and sodium lauryl sulphate 100g per litre in distilled water and for Zym B; fast blue BB 3.5g per litre in 2-methoxy-ethanol.

2.3.2. API ZYM

This is also a gallery of small cupules, the bottom of which forms a support especially designed to contain the enzymatic substrate and buffer. This support allows contact between the enzyme and the generally insoluble substrate. The bacterial suspension medium was sterile distilled water. The galleries were incubated for 4h at 37°C. Zym A and Zym B reagents (described above) were added to the galleries to detect any enzymatic activity.

2.3.3. API RESEARCH ENZYMES

The API ZYM Research kit (API Laboratories, France) allows the

simultaneous study of 90 different enzymes from a single culture (see Appendix 1). These enzymes consisted of esterases (10), osidases (20) and peptidases (60) and were individually distributed amongst small cupules as freeze-dried substrates. The organism suspension medium was 0.1M phosphate buffer pH 7.5, as the enzyme substrates in this test were not buffered. Similarly, as with previous methods, 2 drops of each bacterial suspension was distributed into each cupule. The kits were incubated for 4h at 37°C and were read as follows:-

a) For the osidases, 1 drop of 0.1N sodium hydroxide (NaOH) was added to each cupule, a yellow colouration being positive.

b) The esterases and peptidases were revealed by the addition of 1 drop of Zym A and 1 drop of Zym B to each cupule and allowed to develop for 10 min. The esterases produced a violet colouration when positive, and the peptidases were orange.

All the reactions were exposed to a strong lamp (1000W) for 10 seconds in order to eliminate a yellow base colouration due to an excess of Fast Blue BB that did not react, and to make the negative reaction colourless.

2.3.4. ANTIBIOTIC SENSITIVITY STUDY ON LANCEFIELD GROUP C AND GROUP G STREPTOCOCCI

A small study of antibiotic sensitivities was made on a total of 404 strains of Lancefield group C and group G streptococci of human origin. These were from a variety of sites. They were screened for their antibiotic sensitivity pattern against five antibiotics

by a simple disc diffusion method. These were:-

Penicillin (1 unit)

Chloramphenicol (10 µg)

Tetracycline (5 µg)

Erythromycin (0.5 and 10 µg)

Oxacillin (1 µg)

Strains were grown overnight at 37°C in 5ml of Colindale-Todd Hewitt broth. Blood agar plates were flooded with the overnight broth culture and allowed to dry. The antibiotic discs were then applied and the tests incubated at 37°C for 18h. The sensitivity patterns were read and recorded as follows:-

Sensitive : if the diameter of the zone of inhibition is greater than 10mm.

Resistant : if there is no zone of inhibition.

2.4. GROUPING AND SEROTYPING OF GROUP C AND GROUP G STREPTOCOCCI

The Lancefield group antigen was detected with the Streptex grouping kit (Wellcome Diagnostics) and confirmed when necessary in double gel diffusion by testing Lancefield acid extracts against a series of precipitin grouping sera and with homologous stock antigens as controls.

2.4.1. EXTRACTION OF THE ANTIGENS FOR TYPING

Lancefield acid extracts

0.2M HCL (0.4ml) was added to the cell deposit from 50ml of broth culture, mixed thoroughly and placed in a boiling water bath for 10 min. After cooling and neutralisation with 0.2M NaOH, using

phenol red as the indicator, the extract was centrifuged and the supernate transferred to a fresh tube. The supernate was used as the streptococcal extract for both the group polysaccharide and M-protein antigens but not for the T antigen. Preservative (Thiomersal) was added and the tube was capped and stored at 4°C.

Trypsinised extracts

The streptococci were inoculated into 5ml of Todd Hewitt broth (Difco) with the addition of 0.3ml of a sterile saturated solution of trypsin (Difco 1:250 trypsin) and incubated overnight at 30°C. The culture was then centrifuged and the supernate removed. The pellet was reconstituted in approximately 0.3ml of broth. The concentrated deposit was thus the trypsinised antigenic extract containing the group and T antigens.

2.5. PRODUCTION OF ANTISERA

2.5.1. THE TYPING SERA

The T-typing sera were prepared in rabbits (New Zealand Whites) using, initially, trypsinised whole cell vaccines and subsequently partially purified immunogens (see Section 2.5.2.). Table 4 describes the vaccine strains used for the production of the T antisera. All the strains were deposited in the National Collection of Type Cultures (NCTC). The M-typing sera were prepared in rabbits (New Zealand Whites) using whole cell saline vaccines. The experimental use of purified antigen extracts was also attempted (see Section 2.5.3.). The vaccine strains selected

Table 4

Strains used in the preparation of T antisera

Strain no.	T type	Original PT* no.	Lancefield group
NCTC 4540	7	-	C
NCTC 5370	20	-	"
NCTC 5371	21	-	"
NCTC 11552	200	PT 4225	"
NCTC 11553	201	PT 29	"
NCTC 11564	202	PT 5582	"
NCTC 11565	203	PT 1925	"
NCTC 11629	204	PT 3690	"
	-	PT 1058	"
	-	PT 4279	"
NCTC 5969	16	-	G
NCTC 11554	300	PT 4327	"
NCTC 11555	301	PT 5007	"
NCTC 11556	302	PT 5356	"
NCTC 11557	303	PT 1847	"
NCTC 11566	304	PT 552	"
NCTC 11567	305	PT 5162	"
NCTC 11568	306	PT 1986	"
NCTC 11569	307	PT 739	"
NCTC 11630	308	PT 3623	"

*PT: Provisional type number

Table 5

Strains used in the production of the experimental M antisera

Strain no.	MT type	Source	Lancefield group
NCTC 4540	7	Griffith (1934)	C
NCTC 5370	20	" (1938)	"
NCTC 5371	21	" (1938)	"
NCTC 11564	202	-	"
R86/882	204	Nephritis	"
R85/573	305	Blood	"
R86/273	308	-	"
R83/4496	16	Blood	G
R85/3265	21	Septic arthritis	"
R85/2038	300	Blood	"
R82/2804	301	"	"
R85/2801	302	"	"
R85/2038	303	"	"
NCTC 11630	308	Perineum	"

are listed in Table 5.

2.5.2. PREPARATION OF T VACCINES

Whole cell T vaccines

The method of McLean (1953) was used with some modifications. Each strain was grown in 250ml of Todd Hewitt broth (Media Dept. C.P.H.L.) containing 1% (v/v) of a sterile saturated solution of Trypsin (Difco 1:250, 10% w/v). Incubation was at 30°C for 24h to enhance the production of the T antigen (Elliott 1943). The cells were harvested, washed and resuspended in 25ml of phosphate-buffered saline (pH 7.8, 0.1M saline, 0.2M phosphate) containing 0.5% (v/v) trypsin solution and left at 22°C for 24h. The cells were then washed six times in sterile physiological saline and finally resuspended in 17ml of saline and 3ml of a formalin solution (8% (w/v) formaldehyde in saline) and left at 22°C for 4h. The formaldehyde content of the suspension was reduced after incubation by centrifuging the suspension at 4000 x g for 20 min and replacing half the volume of supernate with saline.

Partially purified T-protein vaccines

The procedure is a modification of the method described by Pakula (1951). Bacterial cultures were grown in 4 litres of Nutrient Broth (Oxoid) supplemented with glucose, 0.5% (w/v) for 72h at 22°C. The cultures were then heated to 70°C for 10 min to kill the cells and then allowed to stand overnight at 4°C. The clear supernate was siphoned off and the remaining suspension centrifuged at 4000 x g for 20 min and then washed twice in physiological

saline. The cells were resuspended in 18ml of 0.1M phosphate buffer pH 7.8 and 2ml of a sterile saturated solution of trypsin 1:250 (Difco) was added. The pH was increased to 8.2 with 1M sodium hydroxide and the mixture incubated at 50°C for 1 hr. After digestion the suspension was centrifuged and the supernate filtered through a sterile cellulose acetate filter, pore size 0.45 µm. For some experiments the supernate was not filtered. The filtrate and unfiltered supernate was acidified to approximately pH 2.5 using 10N HCl to precipitate the fraction containing the T protein. The fluid was stored overnight at 4°C, centrifuged, the supernate discarded and the deposit dissolved in 2ml of 0.1M phosphate buffer pH 7.8. The protein content of the preparation was determined by the method of Lowry et al. (1951).

2.5.3. PREPARATION OF M VACCINES

Whole cell saline vaccines

Each strain was grown in 250ml of Oxoid-Todd Hewitt broth containing Neopeptone (1% w/v) (Ball 1972) and incubated overnight at 37°C. The broth culture was then centrifuged at 4000 x g for 30 min and the cells were harvested and washed three times in saline. They were finally suspended in 18ml of phosphate buffered saline pH 7.8 and heated in a water bath at 60°C for 30 min. The cultures were checked for purity and sterility during the vaccine preparation.

Pepsinised vaccines

A total of 2 litres of Difco-Todd Hewitt broth containing

Neopeptone (2% w/v) was inoculated with 25ml of the broth culture and incubated at 37°C overnight. The cultures were plated for purity and left to stand overnight at 4°C. The cells were harvested and washed twice in ice-cold phosphate buffered saline and once in the extraction buffer (0.067M Na_2HPO_4,KH_2PO_4 buffer at pH 5.8). The cells were finally resuspended in the extracting buffer containing pepsin (Sigma (London) Chemical Co.Ltd.), at a concentration of 50 µg/ml. The cells were then incubated for 30 min at 37°C in a water bath. The reaction was stopped by increasing the pH to 7.5 with 7.5% $NaHCO_3$. The suspension was centrifuged at 8000 x g for 20 min. The cells were extracted again under the same conditions. The supernates were collected and filtered through a sterile cellulose acetate filter (0.45 µm porosity). The filtrates were then pooled and dialysed overnight at 4°C against distilled water. The dialysates were filtered, dispensed in 5ml volumes and finally freeze-dried.

2.5.4. IMMUNISATION PROCEDURE

A single schedule was used for each whole cell vaccine. Each rabbit (New Zealand White) was given a primary intravenous injection of 1ml of the whole cell vaccine, and this was followed two weeks later by a course of twice weekly vaccinations of a 1ml dosage for four weeks, by the same intravenous route.

The partially purified T extract was diluted to 400 µg/ml with saline before inoculation. Comparisons were then made using subcutaneous and intravenous injections of the T-protein

preparation with and without the presence of an adjuvant. The last consisted of an aluminium hydroxide gel (10% v/v of a 2% w/v gel, Serva), prepared by diluting the adjuvant with the protein solution. The pepsin extract was also diluted to 400 µg/ml for use as an immunogen. Comparisons were made using two different routes of inoculation as described previously.

The schedule for the intravenous inoculations of the isolated M antigen was initially similar to the schedule described for the whole cell vaccines. For the subcutaneous inoculations two doses of 0.5ml diluted antigen extract were given in two sites as the primary dose. This was followed by a course of weekly injections of 1ml (0.5ml in each of two sites) for four weeks, two weeks after the primary dose. This was the procedure adopted with aluminium hydroxide as the adjuvant. Freund's complete adjuvant (Difco) was also used with the pepsinised extracts for the stimulation of M antibody response in rabbits. The freeze-dried pepsin extract was reconstituted in saline (0.5ml) and an equal volume of the adjuvant was added. The material was emulsified by sonication and a primary dose of the emulsion given by the intra-muscular route followed by a second dose three weeks later. The final concentration of protein per immunisation was 0.8mg/ml. All the test bleeds (5ml) were made from the ear vein of the rabbit, two weeks after the primary dose and at weekly intervals thereafter. The serum was separated and then clarified by filtration. Each sample was screened for agglutinin and precipitin antibodies to the T or M proteins by slide agglutination and double diffusion in agar

gels (see Sections 2.4.1. and 2.8.4.). The antibody titre was also determined for each bleed by the above methods.

2.5.5. ABSORPTION OF THE ANTISERA

Streptococci grown in 4 litre volumes of Colindale-Todd Hewitt broth for 48h at 37°C were killed by heating at 60°C for 30 min. The cells were centrifuged and washed twice in 40ml of physiological saline and resuspended in 40ml of phosphate buffer (0.1M) pH 7.8.

The antisera were absorbed with a volume of 1:3 of packed cells from an appropriate strain to remove group antibodies (Williams 1958). The absorbing strain used to remove Lancefield group G antibodies was R80/3430 (NCTC 11825) also of group G and carrying the T antigen 25. Antibodies to group C were removed with R79/3540 Lancefield group C with the T antigen 4. The group G strain 2832 carrying the 12M-protein antigen was used for the absorption of the group antibody in the preparation of the M antisera for group G. A representative of S.zooepidemicus NCTC 11824, was used for the group C anti-M sera absorptions. The absorption suspensions were left at room temperature for 24h or at 37°C for 30 min-1h. The absorbed antisera were then titrated against their homologous trypsinised vaccine strain by slide agglutination and then finally against a series of suspensions of known T types at the appropriate dilution previously determined, for cross-reactivity. Ten-fold dilutions of the absorbed antisera were prepared in physiological saline to a dilution of 1 in 10,000.

The titres were determined against their homologous trypsinised vaccine strains. The sera were then used at the highest dilution which produced clear-cut agglutination of the immunising strain. In use all strains of the groups C and G were tested against the available antisera. The M antisera were tested against their homologous vaccine strain by the precipitin reaction in double gel diffusion along with a series of heterologous extracts. All T and M sera were also screened against all the group A type antigens for any cross-reactivity.

Absorption of the antisera with commercial lectin preparations

A commercially prepared lectin gel immobilised with a specific carbohydrate was also used (Bio-Rad Laboratories). As the group determinant for Lancefield group C is N-acetylgalactosamine, the lectin preparation used was 'Affi-Gel-N-acetylgalactosamine'. The efficiency of this method of absorption was compared with conventional methods. The gel was washed twice in phosphate-buffered saline and the fines were decanted, then 2ml of the unabsorbed antiserum was added to the gel slurry (2ml) and incubated for 24-48h at 4°C on a roller machine. The serum was decanted and tested for the presence of group antigen initially by slide agglutination and then by gel-diffusion.

2.5.6. ISOLATION OF IgG FROM RABBIT ANTISERA

This procedure was used to isolate anti-trypsin antibodies and antibodies to the group polysaccharide for use in the affinity chromatography experiments. A total of 25g of ammonium sulphate

$(NH_4)_2SO_4$ crystals were added little by little to 100ml of antiserum. The mixture was left at room temperature overnight. The mixture was then centrifuged at 4000 x g for 30 min. The precipitate which formed was mixed with 25ml of 1.75M $(NH_4)_2SO_4$ and centrifuged. The supernate was discarded. The precipitate was taken up as a suspension in water and dialysed at 4°C for 2 x 12h against distilled water and 1 x 24h against 0.025M sodium acetate, 0.021M acetic acid at pH 5.0, then for 2 x 12h against distilled water and finally against acetate buffer for 24h. The preparation was tested in double gel diffusion to ensure that the antibody was active.

For further purification of the immunoglobulins the supernate from the dialysis procedure was transferred to an ion-exchange column containing 25ml DEAE Sephadex A50, equilibriated with acetate buffer, pH 5.0. The column was eluted with 25ml acetate buffer, pH 5.0 (Harboe and Ingild 1973).

Storage of the antisera

The sera were stored at either -20°C without preservative or at 4°C with thiomersal (20mg/1) as the preservative.

2.5.7. PREPARATION OF CELL WALLS

Cell walls of Lancefield group G streptococci were prepared to determine the amount of rhamnose present for comparison of the rhamnose content in the walls with the content of isolated T-protein preparations.

The cell walls were prepared by growing the streptococci for 48h at 37°C in Todd Hewitt broth. The cell deposit was resuspended in 20ml physiological saline and heated for 30 min at 56°C. The killed cells were then washed twice in distilled water. The washed cells were resuspended in physiological saline (4ml) and were subsequently broken in a Mickle tissue disintegrator. The disintegrated cells were separated from the glass beads by filtration through a sintered glass filter (Jencons grade 17 filter). The walls and any remaining intact bacteria were deposited by centrifugation at 12,000 x g for 10 min. The deposit was washed thrice in sodium dodecyl sulphate (SDS) (1% w/v). Differential centrifugation was then carried out in the last washing solution by centrifuging at 1000 x g for 15 min to deposit the intact cocci and then centrifuging the supernate from the previous centrifugation at 12,000 x g to deposit the cell walls. The cell walls were washed thrice (12,000 x g for 15 min) in distilled water and the preparation was freeze-dried (Pers.comm. G.Colman).

2.5.8. PREPARATION FOR ELECTRON MICROSCOPY

Streptococcal cell walls and isolated T-protein preparations were examined by electron microscopy for the presence of particulate matter which may be related to the group polysaccharide component of the cell wall.

The specimens were applied to formvar-coated grids using a fine

50-dropper and an equal volume of the negative stain (3% phosphotungstic acid) was applied (Brenner and Horne 1959). All preparations were examined by electron microscopy on an AEI EM6B operating at an acceleration voltage of 60kV. The electron microscopy was carried out by Mr A.A.Porter of the Virus Reference Laboratory, Central Public Health Laboratory, London.

2.6. PURIFICATION METHODS FOR T-PROTEIN ANTIGENS

2.6.1. GEL FILTRATION

Preparation of Sephadex G75 and G100

Sephadex G75 and G100 gels (Pharmacia, Uppsala, Sweden) were soaked for 24h at 20°C in 0.01M sodium phosphate buffer, pH 7.6. After sedimentation and removal of the fines by aspiration the gel suspension was evenly packed into an Amicon Wright GA16 column (bed dimensions 24 x 1.6cm). The sample (1ml) was applied, washed twice with its own volume of phosphate buffer at a flow rate of 18ml/h. Fractions (2.5ml) were collected and tested by double gel diffusion for T-precipitin activity. The fractions were also assayed for their protein content (see Section 2.7.1.) and trypsin activity (see Section 2.7.3.).

Calibration of the Sephadex G75 and G100 columns

The void volume of the column was determined by adding a sample of Dextran Blue 2000 (Pharmacia) of molecular weight 2×10^6 to the column and measuring the E_{625} of the eluate. This preparation was totally excluded from the gel. The G75 column was calibrated by means of two standard protein solutions containing 50mg of the

markers. The standard proteins used were bovine albumin (m.wt. 67,000 daltons) and chymotrypsinogen (m.wt. 25,000 daltons) (see Appendix 2).

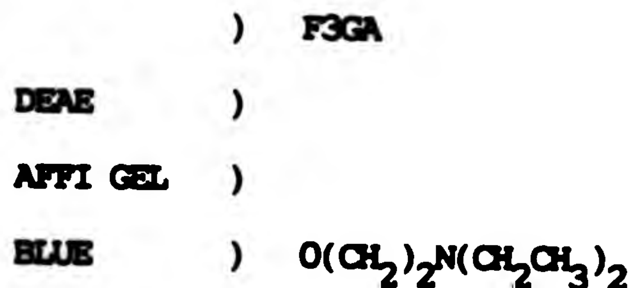
2.6.2. ION EXCHANGE COLUMN CHROMATOGRAPHY

Ion exchange chromatography on DEAE and QAE anionic exchangers

Ion-exchange resins namely DEAE A-50 and QAE A-50 (Pharmacia), were used to purify the crude T-protein preparations. Crude samples were applied to the columns to compare the degree of purification of the crude T-protein preparation. Samples of 1ml were applied to each column and were eluted with a 2M salt gradient made by mixing 2M NaCl and 0.01M sodium phosphate buffer pH 7.6. Samples of eluate of 2 - 3ml were collected (flow rate 0.5ml/min) using an LKB 7000 Ultrorac fraction collector (LKB Ltd., Croydon, Surrey). The optical density of each fraction was determined at 280nm.

DEAE Affi-gel Blue (Bio-Rad Laboratories Ltd)

DEAE Affi-gel blue is prepared by coupling Cibacron(R) Blue F3GA to specially prepared DEAE Bio-Gel(R)A. It was purchased in the coupled form from Bio-Rad Laboratories (Watford, Herts). Primarily this is a beaded, cross-linked agarose which has both Cibacron blue F3GA and diethylaminoethyl groups covalently attached to it.



The gel (15ml) was pre-washed with 5 bed volumes (50ml) of 0.1M CH_3COOH, pH 3.0 containing 1.4M NaCl and 40% (v/v) isopropanol on a sintered glass filter. The pre-wash stage eluted any residual dye from the new preparation. The gel was then washed with 10 bed volumes (100ml) of starting buffer to reduce the ionic strength. The starting buffer contained 0.02M K_2HPO_4 plus NaN_3 (0.2% v/v), pH 8.0. After washing, the gel was transferred to a Pharmacia K9/15 (15 x 0.9cm) column and allowed to settle. It was then eluted with 3 bed volumes of starting buffer (30ml). The sample was thus applied (1ml) and eluted with a 2M salt gradient in starting buffer. The flow rate was 0.3ml/min and 0.6ml fractions were collected and tested by double gel diffusion as in the previous sections. The column was regenerated using 2 bed volumes of 6M guanidine HCL in starting buffer followed by 3 bed volumes of starting buffer alone.

2.6.3. AFFINITY CHROMATOGRAPHY

Coupling of antibodies to CNBr - activated Sepharose(R) 4B

The following antibody coupled gels were prepared:-

i) Anti-trypsin coupled gel for the removal of trypsin from the T-antigen preparations.

ii) Anti-group antibody coupled gel for the removal of the group polysaccharide antigen in crude acid extracts of the protein preparations.

The IgG precipitated antibodies were prepared by the method previously described (see Section 2.5.6.). The coupling procedure

was derived from the Pharmacia handbook, 'Affinity Chromatography'. The coupling gel chosen was cyanogen bromide (CNBr) activated Sepharose 4B (Pharmacia). The reaction of CNBr with Sepharose 4B resulted in a reactive product to which proteins could be coupled under mild conditions by their primary amino groups. The gel was purchased in its active form. CNBr is stated to react with the hydroxyl groups on the Sepharose and thus converts these groups to imidocarbonate groups which then react with the primary amino groups or nucleophiles.

A sample of 2.5g of the gel was swollen and washed for 15 min on a sintered glass filter with 1mM HCL (200ml/g beads) to remove the product stabilisers, dextran and lactose. The gel was then washed with the coupling buffer (0.1M bicarbonate buffer, pH 8.3, containing 0.5M NaCl). The antibody to be coupled (10ml) was then mixed with 30ml of the coupling buffer above and then with the gel suspension. THe mixture was rotated gently end to end, overnight at 4°C. Any unbound material was washed away with coupling buffer and any remaining active groups were reacted with 1M ethanolamine at pH 8.0 for 1 - 2 h at room temperature. Three washing cycles were used to remove any non-covalently bound protein material. Each cycle consisted of a wash at pH 4.0 (0.1M acetate buffer containing 1M NaCl) followed by a wash at pH 8.0 (0.1M bicarbonate buffer containing 1M NACl). The gel was finally washed with phosphate buffered-saline (PBS) pH 7.8. After determining the void volume using blue dextran, a 1ml sample was applied to the column. The degree of coupling of the antibody to the gel product

was determined by measuring the absorbance of the antibody solution at 280nm before and after coupling.

Coupling of trypsin inhibitor

This preparation was used in an attempt to free the crude T protein from trypsin and 25mg of trypsin inhibitor (Sigma Chemical Co.) was coupled to CNBr-activated Sepharose 4B as described previously. The protein was eluted with PBS.

Coupling of crystalline trypsin

Trypsin (250 Kilo units) (Koch-Light) was coupled to 2.5g of CNBr-activated Sepharose 4B as described previously and used in a batch adsorption procedure, as another possible means of digesting streptococcal cells for the release of the T-protein antigen. Some 12ml of concentrated, heat-killed and washed streptococcal cells from 4l of broth culture were added to the gel preparation and digested at 37°C for 2 - 18h. Samples were removed at 2, 4, 8 and 18h. These were dialysed overnight at 4°C against distilled water. They were then freeze-dried to concentrate the protein material and tested for T-protein activity and group activity by gel diffusion. The digests were also screened for trypsin activity.

The affinity columns were regenerated and re-used by the dissociation of the antigen or antibody from the column after use. A 3.5M sodium thiocyanate solution (28.4g per 100ml of NaSCN) at pH 6.8 was passed through the column. The column was washed with

phosphate buffered saline containing sodium azide until it was free of the thiocyanate. The presence of any thiocyanate was examined by taking a test tube with 5ml distilled water to which was added 4 drops of pyridine. Chloroform (2ml) was added to this, followed by a few drops of the neutral thiocyanate solution. The mixture was shaken vigorously. If thiocyanate was present (limit 1 part in 50,000) the chloroform acquired a green colour $[Cu[C_5H_5N]_2(CNS)_2]$ which was water soluble.

2.6.4. HYDROXYLAPATITE COLUMN CHROMATOGRAPHY

The T-protein preparation was applied to a column (30 x 25cm) of calcium hydroxylapatite (HAP). The HAP gel (Bio-Gel HTP, Bio-Rad Laboratories, prepared by the method of Tiselius et al. 1956), was rehydrated with starting buffer, 0.01M sodium phosphate buffer, pH 6.7 with gentle swirling, (1 part HTP to 6 parts starting buffer). The slurry was allowed to settle for at least 10 min and the fines were decanted. This washing procedure was repeated. A sample of 10ml was applied to the column and the protein was eluted with 0.01, 0.1 and 0.3M sodium phosphate buffer at pH 6.7 at a flow rate of 1.5ml/min.

2.6.5. BATCH ADSORPTION

In an attempt to obtain T-antigen free of trypsin, streptococcal cells were digested by batch adsorption using insoluble trypsin linked to polyacrylamide (Sigma Chemical Co.). Altogether 122mg of the preparation were suspended in 10ml distilled water and left at $0^{o}C$ for 2h. The gel was then filtered through a GF/A glass

microfibre filter (Whatman). The suspension was then washed thrice in distilled water. The gel suspension was finally resuspended in PBS and stored with thiomersal as preservative at $0^{o}C$ until used. For the adsorption procedure, the gel suspension was added to 5ml of cells (washed and heat-killed from 4l of broth culture). The cells were digested at $37^{o}C$ at varying time intervals. The tube containing the suspension was shaken end over end during the digestion.

2.6.6. CONCENTRATION METHODS

The protein antigens were concentrated when necessary using the following methods.

Polyethylene glycol 20,000

The cell extracts, broth supernates, chromatography fractions were concentrated ten-fold in dialysis bags suspended in polyethylene glycol 20,000 (BDH Chemicals Ltd., Poole, Dorset), at room temperature for approximately 30min - 1h depending on the initial volume of material in the dialysis bag.

Ammonium sulphate fractionation

Solid ammonium sulphate $(NH_4)_2SO_4$ was added to measured volumes of material. After standing at $4^{o}C$ for 24h, the precipitate was collected by centrifugation and dissolved in the appropriate volume of distilled water or buffer. Each preparation was dialysed against distilled water at $4^{o}C$ for 48h.

Ethanol precipitation

One volume of ice-cold ethanol was used to concentrate four-volumes of the T and M-extracted protein materials at 4°C overnight.

Freeze drying

Standard aliquots of extracted protein material were further concentrated by freeze drying. Measured volumes (1-5ml) of material with known protein contents (previously determined) were dispensed (after dialysing overnight against distilled water) into freeze-drying vials. The dried material was reconstituted into 10% of the original volume.

2.7. BIOCHEMICAL ASSAY METHODS

2.7.1. ESTIMATION OF PROTEIN

The protein content of the fractions was determined by the method of Lowry et al. (1951). The extinction at 750nm of the sample tubes (0.2ml sample, 2.0ml reagent C prepared from 1ml 0.5% (w/v) copper sulphate in 1% (w/v) sodium citrate solution and 50ml of 2% (w/v) sodium carbonate in 0.1M sodium hydroxide solution, plus 0.1ml Folin and Ciocalteu's reagent (BDH, Poole, Dorset) was read, after incubation for 2h at room temperature, against a blank containing 0.2ml of the appropriate buffer in place of the test sample. Protein contents were calculated from a standard curve (E_{750} vs µg protein/ml) for bovine serum albumin (see Appendix 2).

2.7.2. ESTIMATION OF RHAMNOSE

The degree of cell wall contamination in protein preparations was determined by the estimation of rhamnose in the samples following trypsin digestion by the method of Gibbons (1955). Glass tubes were placed in an ice bath and chilled sulphuric acid (concentrated H_2SO_4, 6 parts acid and 1 part water) was added slowly to each tube containing the samples and to a series of standards. These were shaken and left to stand at room temperature. These were then placed in a boiling water bath for 10 min and then immersed in cold water. Mercaptoacetic acid (0.1ml) was added to each tube and the mixture was allowed to stand in the dark for 3h. The optical density (O.D.) of each mixture was read at 400 and 430nm. The absorbance at 430nm was subtracted from that at 400nm and the % rhamnose content was then 4 calculated from a calibration curve (see Appendix 2).

2.7.3. DETERMINATION OF TRYPSIN ACTIVITY

The proteolytic activity of the trypsin was determined by placing drops of serial dilutions of the solution on exposed photographic film. This was incubated in a moist chamber at 37°C for 1h then washed in running water. The titre was the highest dilution producing a clear area on the film, and was not less than 1 in 10^4 for use.

2.8. DETECTION OF T AND M-PROTEIN ANTIGENS

2.8.1. GRADIENT POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF T PROTEIN IN THE PRESENCE AND ABSENCE OF SODIUM DODECYL SULPHATE (SDS)

Preparation of gels

These were commercially prepared gradient gels (Universal Scientific Ltd, Woodford Green, Essex), which consist of a continuous polyacrylamide gradient. The gradients used were 2.5 - 28% (T) and 2.5 - 40% (T). The gel slab size was 75 x 75 x 2.8mm. Before use, the gels were equilibriated at room temperature for 2 - 3h, and then in the electrophoresis buffer at 70V for 30 min. The buffer contained 0.9M tris-hydroxymethylmethylamine (Tris), 0.8M boric acid and 0.0025M Na_2EDTA, at pH 8.4. Sodium phosphate buffer 0.055M containing SDS 0.1% (w/v), at pH 7.0 (Weber and Osborn 1969) was also used as an additional buffer system.

Preparation of samples

The following standard proteins (Pharmacia) were used: thyroglobulin (mol.wt. 66.9 x 10^4 daltons), ferritin (44 x 10^4 daltons), catalase (23.2 x 10^4 daltons), lactate dehydrogenase (14 x 10^4 daltons) and albumin (6.7 x 10^4 daltons). This high molecular weight (HMW) marker set was used in the native state and in the presence of SDS. A second set containing lower molecular weight proteins (LMW) was used solely for SDS PAGE. The proteins in this mixture were: phosphorylase b (9.4 x 10^4 daltons), albumin (6.7 x 10^4 daltons), ovalbumin (4.3 x 10^4 daltons), carbonic anhydrase (3.0 x 10^4 daltons), trypsin inhibitor (2.01 x 10^4

daltons) and α-lactalbumin (1.44×10^4 daltons). For PAGE, 100 µl of the appropriate buffer was added to each sample and marker. In the presence of SDS, 100 µl of buffer containing SDS 2.5% (w/v) and β-mercaptoethanol (BDH) 5% (v/v) were added and the samples were heated at 100°C for 5 min. Samples of 10-15 µl were applied to the gel and were left to migrate into the gel at 80V and finally allowed to electrophorese at 100V overnight.

Protein staining after gradient gel electrophoresis

After electrophoresis, the proteins were fixed in 10% (w/v) sulphosalicyclic acid for 30 min. The staining solution contained Brilliant Blue R (0.1% w/v, Sigma Chemical Co.), in methanol (25% v/v), acetic acid (10% v/v) and water (v/v). The gels were stained at room temperature overnight followed by destaining in methanol (25% v/v), acetic acid (10% v/v), water (65% v/v) for approximately 2 days. The gels were stored in 7% (v/v) acetic acid.

2.8.2. SDS-PAGE

As results using the mini-gradient gel system were not consistent, it was decided to explore the use of homogeneous polyacrylamide gels to investigate the molecular nature of the T and M-protein antigens.

Preparation of gels

The purification and extraction procedures were monitored by electrophoresis in 10% and 12.5% polyacrylamide slab gels in the

daltons) and α-lactalbumin (1.44 x 10^4 daltons). For PAGE, 100 µl of the appropriate buffer was added to each sample and marker. In the presence of SDS, 100 µl of buffer containing SDS 2.5% (w/v) and β-mercaptoethanol (BDH) 5% (v/v) were added and the samples were heated at 100°C for 5 min. Samples of 10-15 µl were applied to the gel and were left to migrate into the gel at 80V and finally allowed to electrophorese at 100V overnight.

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Preparation of gels

The purification and extraction procedures were monitored by electrophoresis in 10% and 12.5% polyacrylamide slab gels in the

presence of SDS 0.1% (w/v), using a discontinuous buffer system at pH 8.8 (Laemmli 1970). The gel buffer contained 15g Tris, 72g glycine and 5g SDS per litre of water, pH 8.3. An acrylamide solution (BDH Chemicals Ltd., 'Electran'), containing 30g of acrylamide and 0.8g bis-acrylamide (BDH, 'Electran') was dissolved in distilled water to give 100ml, was filtered through a Whatman no.1 filter paper and stored at 4°C in a dark bottle. The stacking gel buffer was 0.5M Tris-HCl, pH 6.8 and the resolving gel buffer 1.5M Tris-HCl, pH 8.8. Electrophoresis was carried out on 12.5% polyacrylamide gels with a 5% stacking gel, at a constant current of 30mA per gel for approximately 3h at 10°C. The HMW and LMW (Pharmacia) markers described earlier were used.

Preparation of samples

Samples and standards were diluted with an equal quantity of sample buffer containing SDS 2% (w/v), β-mercaptoethanol 5% (v/v), glycerol 10% (v/v), bromophenol blue 0.05% (w/v), and were heated to 100°C for 5 min before applying to the gel.

Protein staining after SDS-PAGE

The gels were stained in Brilliant Blue R (Sigma Chemical Co.) 0.1% (w/v), in methanol 25% (v/v), acetic acid 10% (v/v) and water 65% (v/v) at room temperature overnight. They were destained in 25% methanol (v/v) acetic acid 10% (v/v) and water (v/v) over a period of two days and were stored in 1% (v/v) acetic acid prior to drying.

The other procedure used was the silver staining method of Hempelman et al. (1984). The gel was soaked in 200ml of each of the following solutions sequentially. The gel was prefixed in trichloroacetic acid 10% (v/v) for 30 min, and was then soaked in ethanol/acetic acid/water (20/5/75% v/v) containing dithiothreitol 0.002% (w/v) for 30 min. The gel was then immersed in potassium dichromate 0.5% (w/v) for 5 min, followed by soaking in water for 5 min. The gel was immersed in silver nitrate 0.1% (w/v) for 10 min followed by 1 min in water. It was then transferred to a freshly prepared solution of sodium carbonate 3% (w/v) containing paraformaldehyde 0.02% (w/v) for 7 min. For storage, the gels were left in 1% acetic acid (v/v) solution.

Preparation of the calibration curve

The relative migration values (Rf) for the standard proteins were determined as follows:-

$$Rf = \frac{\text{distance protein has migrated from origin}}{\text{distance from origin to reference point}}$$

A calibration curve was determined by plotting the Rf values of the standards against the logarithm to base 10 of their corresponding molecular weights (see Appendix 2). The molecular weight of the unknown protein was determined by:-

i) Measurement of the migration distance of the protein.

ii) Calculation of the Rf for the protein.

iii) Location of the point on the calibration curve which

corresponds to the Rf value for the protein of unknown molecular weight. The value on the logarithmic scale which corresponds to this point is the estimated molecular weight of the protein (see Appendix 2).

This method was applied to both the gradient and homogeneous polyacrylamide gels.

Densitometry

Densitometry tracings of gels were carried out using the LKB 2202 UltroScan Laser Densitometer and the Bio-Rad Video Densitometer, model 620.

2.8.3. ISO-ELECTRIC FOCUSING (IEF) IN THIN LAYER POLYACRYLAMIDE GELS

This technique was used to determine the iso-electric point of the T protein. Commercially available ampholine polyacrylamide (PAG) gel plates were used (LKB 1804 Ampholine, PAG plates). Each gel was 1mm thick, polymerised on a thin plastic sheet, and contained Ampholine carrier ampholytes (2.4%). Cross-linking (C) was given as 3% and the gel concentration (T) 5%. The pH range of the PAG plate was 3.5 - 9.5. The samples were dialysed against glycine 1% (w/v) overnight at 4°C prior to focusing. The electrode strips were soaked in 1M H_3PO_4 to form the anode and 1M NaOH (cathode) before applying them to the gel surface. The samples were applied onto filter paper strips that were then transferred to the gel surface. The gels were focused for approximately 3h in an LKB 2117 Multiphor. The voltage was increased until a maximum of 800V

was reached. The pH gradient of the gel was measured using a surface electrode. The proteins were subsequently fixed for 30 min in an aqueous solution of sulphosalicylic acid (3.45% w/v) and trichloroacetic acid (15.5% w/v). This solution fixes the proteins in position and allows the Ampholine carrier electrolytes to diffuse out. The gel was then washed and brought to the correct pH for staining, by placing it in destaining solution (ethanol 25% (v/v), acetic acid 8% (v/v) in water (v/v)) for 5 min. The gel was then stained for 10 min in staining solution which had been preheated to 60°C (0.460g Coomassie Brilliant Blue R-250 in 400ml destaining solution). The gel was then destained until the background was clear and finally preserved in glycerol 4% (v/v).

2.8.4. DOUBLE GEL DIFFUSION

Agarose (1% w/v),(Koch-Light, Colnbrook, UK), was dissolved in phosphate buffered saline (Oxoid, Code BR 14a, Dulbecco 'A' tablets) containing a final concentration of Thiomersal (0.2% v/v). A thin agar film was applied to 76 x 26mm microscope slides and they were then dried at 37°C overnight. A top layer of agar (4ml) was applied to the base layer. Three patterns of wells each consisting of six peripheral wells around a central well, were cut out with a metal template on each slide. The distance between the periphery of the wells was 3mm. The protein antigens were added to the peripheral wells with antiserum in the centre well. Where possible a stock homologous control antigen was present in one of the peripheral wells. Slides were incubated at room temperature for 18h before examination for precipitin lines.

2.8.5. COUNTERCURRENT IMMUNOELECTROPHORESIS (CIE)

Agarose gels 1% (w/v) were prepared in veronal buffer pH 8.6, ionic strength 0.03. A bridge was formed with six 76 x 26mm slides, each with a 3ml buffered agar overlay and 0.01ml of sample was added to each 2mm diameter central well before electrophoresis at 200V for 2h. A longitudinal trough was then cut alongside the central well and the antiserum was added and allowed to react with the antigen for 24h at room temperature. Antigen-antibody combinations were visible as a series of one or more precipitin lines (Grabar and Williams 1953).

2.8.6. CROSSED IMMUNOELECTROPHORESIS

A 10ml layer of 1% (w/v) agarose in veronal buffer (pH 8.2, ionic strength 0.02)was poured onto a non-precoated glass slide (80 x 80mm). A hole was bored 1cm from the edge of the slide and 5 - 10 µl of sample was applied. The antigen was electrophoresed at 200V for 2h. An area of 60 x 80mm of agar was removed from the slide and replaced with an antibody-containing agarose gel in veronal buffer (8ml agar and 0.5ml IgG precipitated antibody). Electrophoresis was continued in the second dimension for 18h at 100V. The slides were stained in Coomassie Blue R 0.025% (w/v) in methanol/acetic acid/water as described above.

2.8.7. ROCKET IMMUNOELECTROPHORESIS

A layer of agarose 1% (w/v) containing antibody (9.8ml agar and 0.2ml serum) was poured onto a slide (80 x 80mm) precoated with

agarose in veronal buffer. Seven wells were bored 1cm apart and 1.5cm apart from one end of the slide. Serial dilutions of antigen to 1 in 64 were placed into the appropriate wells (10 µl per well). The gels were then electrophoresed at 100V and 6mA for 18h. The slides were stained as above to demonstrate the precipitin lines.

2.8.8 WESTERN BLOTTING

For Western blotting, proteins resolved by SDS-PAGE were transferred electrophoretically to nitrocellulose membranes (Gelman Sciences Inc. U.S.A.) according to the method of Towbin *et al.* (1979) with a transfer time of 18h at 9A and constant voltage 30V. The electrophoresis buffer was 25mM Tris-HCl, 1.92mM glycine and 20% (v/v) methanol per litre of water at pH 8.3. For confirmation of protein transfer, the membrane was stained with amido black (0.1% w/v) in methanol (45% v/v), acetic acid (10% v/v) and water.

2.8.9. IMMUNOBLOTTING

Horseradish peroxidase assay

After transfer the unstained nitrocellulose membrane was incubated for 30 min at room temperature in 10mM Tris-HCl buffer, pH 7.4, containing bovine serum albumin 3% (w/v) and sodium chloride 0.9% (w/v) to block the remaining protein binding sites. Rabbit anti-T antibody diluted in the same buffer was applied to the membrane and allowed to react for 2h at room temperature. The membrane was then washed five times with intermittent 5 min incubations in Tris-buffered saline (TBS), (10mM Tris in saline, pH 7.4) at 4°C.

Peroxidase conjugated goat anti-rabbit IgG antibody (Tago, Inc. Burlingame, California, U.S.A.) diluted 1 in 2000 in TBS with bovine serum albumin 3% (w/v) and Nonidet P40 0.05% (v/v)) was applied to the membrane and incubated for 2h at room temperature. After incubation, the membrane was washed five times as above. The reaction was developed by the addition of enzyme substrate solution which was prepared by adding to 100ml TBS containing 60 µl of ice-cold hydrogen peroxide 30% (v/v) and 60 mg of Horseradish peroxide (HRP) colour development reagent containing 4-chloro-1-napthol (Bio-Rad) in 20 ml of ice-cold methanol. The reagent was prepared and used immediately. The reaction required at least 30 min at room temperature. After development, the reaction was stopped by rinsing with distilled water. Membranes were dried at room temperature.

Alkaline phosphatase assay

Following protein transfer the membrane was placed into the blocking solution (20mM Tris, 500mM sodium chloride, pH 7.5 containing gelatin 3% (w/v) (3% gelatin-TBS) for 30 min at room temperature. The membrane was then washed twice in 20mM Tris, 500mM sodium chloride containing Tween-20 0.05% (v/v), pH 7.5 (TTBS) and then transferred to a dish containing the first antibody diluted accordingly in 3% gelatin-TBS. This was incubated for 2h with gentle agitation. Any unbound antibody was removed by washing the membrane twice in TTBS for 5 min. The nitrocellulose was then transferred to the second antibody conjugated to alkaline phosphatase (Bio-Rad) and was incubated for 1h. The membrane was

then washed in TTBS as before and was then developed in 5-Bromo-4-chloro-3-indoyl phosphate toluidine salt (BCIP), p-nitro blue tetrazolium chloride (NBT) Alkaline Phosphatase colour development solution (Bio-Rad). Tween-20 was used in all washing procedures. This was essentially to eliminate the overall background and non-specific hydrophobic reactions. The development solution was prepared immediately prior to use, as follows: 1 ml of 70% (v/v) N, N-dimethyl-formamide (DMF) solution was prepared by mixing 0.7 ml DMF with 0.3 ml of water. 30 mg of NBT was dissolved in this solution. This was solution A. In a second vial, 15 mg of BCIP were dissolved in 1 ml DMF. This was solution B. Prior to starting the colour development procedure solutions A and B were added to 100 ml of carbonate buffer (0.1M $NaHCO_3$, 1.0mM $MgCl_2$, pH 9.8) and the solution was used immediately. The final use concentrations for the development were 0.3 mg/ml for NBT and 0.15 mg/ml for BCIP. The membrane was immersed in this solution for at least 15 min. The reaction was stopped by immersing the membrane in distilled water for 10 min and was dried as described above.

2.9. DETECTION OF M PROTEINS

2.9.1. DIRECT BACTERICIDAL TEST

The ability of group C and group G streptococci to survive and multiply in normal human blood was investigated by adding viable organisms to fresh heparinised human blood. A 3h culture of the organism in Difco-Todd Hewitt broth with Neopeptone 2% was diluted

so that 0.02 ml contained 50-100 colony forming units (5 x 10^3 organisms/ml). Culture dilutions were made in saline containing Difco-Todd Hewitt broth 5% (v/v) with Neopeptone. A 0.02 ml sample of the diluted culture was inoculated into 0.3 ml of fresh human blood containing 5 units/ml heparin. The tubes were sealed and incubated for 3h at 37°C on a roller machine so that end over end mixing occurred. Several donors were used for each strain tested. After 3h, 0.02 ml samples were placed into 9cm sterile petri dishes containing 0.5 ml of broth saline. After mixing, blood agar (approximately 20 ml of 5% horse blood (v/v) in nutrient yeast agar) was poured into the plate. The plates were incubated at 37°C for 18h and the haemolytic colonies counted. The inoculum size was calculated from a viable count made at the start of the test.

2.9.2. BACTERICIDAL NEUTRALISATION TEST

Strains previously tested for their ability to survive in the donor blood, were grown in Difco Todd-Hewitt broth supplemented with Neopeptone for 18h. Subcultures were made as described above in broth for 3h at 37°C. Thousand-fold dilutions were made in broth saline and 0.02 ml samples were placed into the appropriate tubes. Antiserum (0.02 ml) homologous or heterologous were added and control tubes containing normal rabbit serum or broth saline were also included. Fresh normal human blood (0.3 ml) containing 5 units/ml of heparin was then added. Pour plates of each culture dilution were prepared to allow the determination of the initial inoculum. The tubes were sealed and incubated at 37°C on a roller

machine. After 3h, samples (0.02 ml) were placed into petri dishes containing 0.5ml broth saline. Pour plates were prepared and incubated at 37°C overnight. The haemolytic colonies were then counted.

2.9.3. TYPE-SPECIFIC COMPLEMENT FIXATION TEST

The detection of antibodies to the M-associated protein (MAP) in human sera is a confirmatory test for a recent infection and indicative that an M protein-like antibody is present. A complement fixation text (CFT) was used to detect these antibodies. The highest titre of the antigen extract at which complement fixation occurs with a standard dose of anti-MAP serum in an immunological system gives an indication of the amount of M protein produced by such a strain. The complement fixation method described by Bradstreet and Taylor (1962) was employed and modified according to Widdowson et al. (1971) to a microtitre technique for MAP tests. The antisera used in the test were normal human sera and sera from patients who had had group C or group G streptococcal infections. Lancefield acid extracts of known serotypes of group C and group G were used as antigens. Heterologous extracts and antisera were included in the test to confirm that the fixation of complement was the result of interaction between type specific antigen and antibody. The dose of complement (Wellcome) was three times the minimum haemolytic dose (3MHD) which was predetermined by titrating haemolytic serum (horse anti-sheep serum, Wellcome) against complement to determine the optimal concentration of the haemolytic serum and the titre of the complement. The antigen

extracts were diluted to 1 in 320 as was a standard known positive extract of M-type 30 (Lancefield group A). Fixation was allowed to occur at 4°C overnight. The sera were inactivated at 56°C for 30 min to neutralise any complement activity prior to use. After incubation at 4°C the plates were prewarmed at 37°C for 30 min and sensitised sheep red blood cells were added. The tests were then incubated at 37°C for 30 min with shaking at 15 min and 30 min. After 2h at 4°C, the results were recorded as, 4+:complete fixation, 3+:75% fixation, 2+:50% fixation, 1+:25% fixation and 0: complete haemolysis.

3. RESULTS

3.1. BIOCHEMICAL AND ENZYME PROFILES OF LANCEFIELD GROUP C AND GROUP G STREPTOCOCCI

A total of 100 isolates of Lancefield group C and group G streptococci were examined and tested for exoenzyme production and subjected also to a range of biochemical tests. These included the one species of group G from humans which currently remains nameless, the one species of group G from animals which has been designated as S.canis, and the four named species of group C from similar diverse sources. Also included were strains of S.milleri possessing the Lancefield group C or group G antigen, isolated from human infections. The species examined are described in Table 6.

3.1.1. IDENTIFICATION OF GROUP C AND GROUP G STREPTOCOCCI BY API 20 STREP

All strains formed phosphatase, leucine aminopeptidase, hydrolysed arginine but did not ferment arabinose, mannitol or inulin nor hydrolyse hippurate or form α-galactosidase or pyrrolydonylarylamidase (Table 7). The remaining reactions varied according to the species. Many of the fermentation reactions exhibited variability within the same species. Therefore, it was not possible, using this kit alone, to distinguish between the human and animal S.equisimilis isolates on one hand and the human isolates of group G on the other. The animal strains of S.equisimilis (group C) fermented the sugar glycogen whereas all the human isolates did not. This was perhaps a significant difference. The dog and cat isolates of group G were virtually identical biochemically and could not be distinguished. These

Table 6

Lancefield group C and group G streptococci examined by biotyping and enzyme profile tests

Species	No. of isolates	Lancefield group	Source
S.equisimilis	25	C	Human (H)
S.equisimilis	3	C	Animal (A)
S.dysgalactiae	6	C	A
S.equi	5	C	A
S.zooepidemicus	8	C	H and A
Streptococcus sp.	25	G	H
*Streptococcus sp.	10	G	Dog
*Streptococcus sp.	3	G	Cat
S.milleri	8	C	H
S.milleri	7	G	H

*Designated S.canis

Table 7

Identification of group C and group G streptococci using the API 20 STREP Kit

Species	Lanc group	VP	AESC	BGUR	BGAL	RIB	SOR	LAC	TRE	RAF	AMD	GLY
S.equisimilis (H)	C	-	+	+	-	+	-	v	+	-	+	-
S.equisimilis (A)	C	-	+	+	-	+	-	-	+	-	+	+
Streptococcus sp. (H)	G	-	+	+	-	+	-	-	+	-	+	-
Streptococcus sp. (D)	G	-	+	-	+	+	v	+	v	-	+	-
Streptococcus sp. (C)	G	-	+	-	+	+	-	+	-	-	+	-
S.zooepidemicus	C	-	+	+	-	+	+	+	v	v	+	+
S.equi	C	-	+	+	-	v	-	v	-	-	+	+
S.dysgalactiae	C	-	-	+	-	+	v	+	+	-	+	v
S.milleri	C	+	+	-	-	-	+	+	+	-	+	-
S.milleri	G	+	+	-	-	-	v	+	+	v	v	-

Key

v: Variability within same species

VP: acetoin production
AESC: aesculin hydrolysis
BGUR: beta glucuronidase
BGAL: beta galactosidase
RIB: fermentation of ribose
SOR: " " sorbitol
LAC: " " lactose
TRE: " " trehalose
RAF: " " raffinose
AMD: " " starch
GLY: " " glycogen

(H): Human
(A): Animal
(C): Cat
(D): Dog

All PAL,LAP,ADH positive

PAL: Alkaline phosphatase
* LAP: Leucine arylamidase
ADH: Arginine dihydrolase

INU,MAN,ARA,PYRA,α GAL negative

MAN: Mannitol
ARA: Arabinose
PYRA: Pyrrolidonylarylamidase
α GAL: α galactosidase

* LAP : Leucine arylamidase or aminopeptidase

strains, however, fermented lactose whereas the human strains did not. Overall, it was not possible to establish any differentiation based on fermentation patterns, with particular reference to the fermentation of trehalose, lactose or raffinose and aesculin hydrolysis. Differentiation based on these sugars has been previously described by other workers to distinguish human and animal group G strains (Biberstein et al. 1980, Clark et al. 1984). Two isolates from cases of endometritis in dogs were unusual in their production of α-galactosidase.

3.1.2. DIFFERENTIATION OF GROUP C AND GROUP G STREPTOCOCCI USING ENZYME PROFILES

The use of the API ZYM system allowed the simultaneous study of initially 19 enzymes (the list of enzymes is given numerically in the Appendix (1), these were given the numbers 61-80, with 61 being the negative control).

Six of these enzymes appeared to be common to all group C and group G streptococci, these were alkaline phosphatase, the esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and valine arylamidase. Of the enzymes detected and produced by these organisms, six were useful as distinguishing markers (Table 8). However, it was not possible to differentiate between human isolates of S.equisimilis and human isolates of group G. Also, it was not possible to differentiate between cat and dog isolates of group G. However, these strains could be differentiated from the human isolates of group G by the production of the enzymes

Table 8

Species differentiation of group C and group G streptococci using the API ZYM system

Species	Group	ENZYMES					
		68	70	72	74	75	76
S.equisimilis (H)	C	+	-	+	-	+	+
" " (A)	C	+	-	+	-	+	+
Streptococcus sp.(H)	G	+	-	+	-	+	+
" " (A)	G	+	+	+	+	-	+
S.zooepidemicus	C	+	+	+	-	+	+
S.equi	C	+	+	+	-	+	+
S.dysgalactiae	C	-	-	+	-	+	+
S.milleri	C	+	+	-	-	-	-
S.milleri	G	v	+	-	-	-	+

68 Cystine arylamidase

70 Chymotrypsinogen

72 Naphthol-AS-BI-phosphohydrolase

74 β-galactosidase

75 β-glucuronidase

76 α-glucosidase

chymotrypsin and β-galactosidase. Examples of enzyme reactions obtained are shown in the Appendix (1).

Strains of S.milleri with the Lancefield group C antigen could be differentiated from S.milleri strains with the G antigen by this system. However, this system and the API 20 STREP kit did not successfully distinguish between the two species S.equi and S.zooepidemicus. As both these species are β-haemolytic, differentiation is very important. But, strains of S.dysgalactiae could be distinguished from the other species of Lancefield group C based on these tests (Table 8).

To investigate further the identification of group C and group G streptococci by enzyme profile patterns, all strains were tested against a battery of 90 enzymes available as a research kit system from API. These enzymes are listed as 1-60 and 81-100 in the appendix. Enzymes 1-60 produced reproducible consistent results, whereas major discrepancies and inconsistencies of reactions were obtained with enzymes 81-100. These enzyme substrates (81-100) were not useful in the differentiation of these Lancefield groups. Of the 60 enzymes investigated, seven were found to be extremely useful for the identification and differentiation of the species (Table 9). The results from the battery of 60 substrates were combined with the results obtained from the API ZYM tests and nine very useful enzyme substrates emerged (Table 10). Based on these enzymes, it was possible to differentiate between:

a) All species of Lancefield group C and group G streptococci

Table 9

Subdivision of group C and group G strains based on seven additional substrates

Species	ENZYMES 15	23	36	39	42	49	52
S.equisimilis (H)	+	+	-	-	+	+	-
" " (A)	+	-	-	-	+	+	-
Streptococcus sp.(H)	+	-	-	-	+	+	-
" " (Dog)	+	-	-	-	+	v	v
" " (Cat)	+	-	+	+	-	-	-
S.zooepidemicus	-	-	+	+	-	-	-
S.equi	+	-	+	+	-	-	-
S.dysgalactiae	+	-	+	-	+	+	-
S.milleri C	v	-	-	-	-	+	+
S.milleri G	-	-	-	-	v	+	+

15 Glycyl-glycine arylamidase

23 α-L-glutamate arylamidase

36 α-L-aspartyl-L-alanine arylamidase

39 α-L-glutamyl-L-histidine arylamidase

42 Glycyl-L-tryptophane arylamidase

49 L-phenylalanyl-L-arginine arylamidase

52 L-prolyl-L-arginine arylamidase

Table 10

Enzyme differentiation of Lancefield group C and group G streptococci isolated from human and animal infections

Species	Group	ENZYMES 1 (15)	2 (23)	3 (42)	4 (52)	5 (70)	6 (72)	7 (74)	8 (75)	9 (76)	Enzyme Profile
S.equisimilis(H)	C	+	+	+	-	-	+	-	+	+	1.2.3.6.8.9
" " (A)	C	+	-	+	-	-	+	-	+	+	1.3.6.8.9
Streptococcus sp. (H)	G	+	-	+	+	-	+	-	+	+	1.3.4.6.8.9
Streptococcus sp. (A)	G	+	-	+	-	+	+	+	-	+	1.3.5.6.7.9
S.zooepidemicus	C	-	-	-	-	+	+	-	+	+	5.6.8.9
S.equi	C	+	-	-	-	+	+	-	+	+	1.5.6.8.9
S.dysgalactiae	C	+	-	+	-	+	+	-	+	+	1.3.5.6.8.9
S.milleri	C	+	-	-	+	+	-	-	-	-	1.4.5
S.milleri	G	+	-	+	+	+	-	-	-	+	1.3.4.5.9

Enzymes

1. Glycyl-glycine arylamidase
2. α-L-glutamate arylamidase
3. Glycyl-L-tryptophane arylamidase
4. L-prolyl-L-arginine arylamidase
5. Chymotrypsin
6. Naphthol-AS-BI-phosphohydrolase
7. β-galactosidase
8. β-glucuronidase
9. α-glucosidase

including S.milleri.

b) Human and animal isolates of S.equisimilis and human isolates of group G.

c) S.milleri strains with group C or group G antigens.

Experimental kits incorporating these nine enzyme substrates were kindly prepared by Dr Daniel Monget, API Laboratory Products Ltd., France and Dr Brian Thomas, API, U.K. The identical 100 strains were retested with the kit incorporating the nine enzyme substrates. Results obtained with all strains were consistent and reproducible.

Summary

The use of these nine enzyme substrates as a rapid screening kit could prove to be useful both clinically and epidemiologically as results are apparent within 4h. Full biochemical identification or even group serology may not even be necessary.

The final results based on enzyme detection using nine substrates differentiated between all species of Lancefield group C and group G streptococci, and between human and animal isolates within the same species. As the majority of streptococcal grouping kits are extremely rapid in producing results, serology in conjunction with a rapid enzyme kit may be a valuable alternative to full identification.

Further differentiation and an understanding of the epidemiology of these organisms would be achieved therefore by serological tests

based on antigenic components of the cell wall, for example, proteins.

3.2. DEVELOPMENT OF THE SEROTYPING SCHEME AND THE PRODUCTION OF ANTISERA TO THE T PROTEIN

This investigation describes a method for the serological subdivision of Lancefield group C and group G streptococci of human origin, based on T-protein antigens associated with their cell walls. Griffith's original subdivision of pyogenic streptococci into different T-types included types 7, 16, 20 and 21 which were later found not to belong to Lancefield group A but to be strains of either group C or group G. Antisera were prepared against these and against strains that did not carry these antigens in order to develop a serotyping scheme capable of identifying Lancefield group C and group G streptococci of human origin. The serotyping system could prove to be useful for the study of random isolates to see whether particular serotypes are associated with certain disease states or whether clusters of a single type occur in possible outbreaks of infection. In these ways the scheme might provide indications of the ability of these streptococci to cause infectious disease and thus aid their recognition as an apparent 'new' pathogen.

3.2.1. T-TYPING SCHEME

The T-protein antigens were identified by the agglutination of

trypsinised suspensions of the streptococci with their homologous antisera. Cell suspensions were prepared by exposure to trypsin (V.D.Allison, unpublished) as modified by Efstratiou (1980). Each antiserum was used at the highest dilution which gave a strong agglutination reaction. This varied between 1 in 500 to 1 in 10,000 in sera prepared using whole cell vaccines and 1 in 5 to 1 in 10 using partially purified immunogens.

Initially the suspensions were screened against five pooled sera. If a reaction was obtained with one of the pools, the individual sera for that pool were examined. The arrangement of the scheme is shown in Table 11. Representatives of group C were initially tested with all five pooled sera and those of group G with the pools numbered 1, 2 and 3. If agglutination was not detected with any of the pools, the cell suspensions were tested with the T-typing antisera for the Lancefield group A streptococci which are known to react with some strains of either group C or group G. The group A, T-sera of particular interest in that set were types 2, 4, 28 and the complex 8, 25, IMP 19. Cross reactivity with the typing sera for group C and group G did not occur with strains of Lancefield group A streptococci (Table 12).

Type numbers

The type numbers 7, 16, 20 and 21 were used by Griffith (1934) for streptococci later found to belong to the groups C and G. It is proposed that new serotypes associated with group C be given numbers in a series beginning with 200 and that numbers commencing

Table 11

Plan of the serotyping scheme for groups C and G streptococci based on the T-protein antigens

		Component sera
Pool 1	Type	7, 21, 301, 302
Pool 2		16, 20, 300, 304, 307
Pool 3		303, 305, 306, 308
Pool 4		200, 202, 203, PT 4279
Pool 5		201, 204, PT 1058

PT = Provisional type number

<u>Table 12</u>

<u>Agglutination reactions of Lancefield group C and group G streptococcal serotypes</u>

Serotype Strains	7	20	21	200	201	202	203	204	1058	4279	16	300	301	302	303	304	305	306	307	308	4860	7023
	A	n	t	i	s	e	r	a														
7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
200	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
201	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
202	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
203	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
204	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1058	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
4279	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
300	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
301	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
302	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
303	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
304	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
305	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
306	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
307	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
308	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
4860	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
7023	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

+: Positive agglutination reaction
-: Negative " "
There were no cross reactions with the T-protein antigens of Lancefield group A

with 300 be used for the group G streptococci. This form of numbering has been accepted by the streptococcus reference laboratories in other countries (Pers.comm.G.Colman).

3.2.2. PRODUCTION OF ANTISERA TO THE T PROTEIN

The established method for the preparation of T-typing sera of Lancefield group A is based on the use of trypsinised whole-cell vaccines. This therefore was the initial method used with Griffith's T-types 7, 20, 21, (Lancefield group C) and 16, (Lancefield group G) to determine the immunogenicity in rabbits to these T proteins. A total of 110 rabbits were used for the complete study. Whole cell vaccines were used to immunise 58 rabbits, and the remaining 52 were immunised with partially purified vaccines (Table 13).

Antisera were initially prepared for the T antigens 7, 16, 20 and 21 and their occurrence in human strains of Lancefield group C and G was confirmed by slide agglutination. These sera agglutinated only one-fifth of the strains tested and rabbits were then immunised with strains that did not carry these antigens obtained from random clinical isolates. In this way a provisional set of 19 experimental sera were produced.

Rabbits were bled at weekly intervals and the test bleeds for each type were studied individually for the presence of group specific antibodies and the presence of T-protein antibodies by slide agglutination and double gel diffusion.

Table 13

Immunisation of rabbits for the production of antisera to the T protein

No. of rabbits	Type of vaccine	Route of inoculation
30	whole-cell trypsinised	†IV
28	whole-cell saline	IV
*33	T extract	IV ‡SC SC + IV
*19	Pepsinised extract	SC SC + IV

* These vaccines were used with and without the presence of the adjuvant (aluminium hydroxide).

†IV: Intravenous route

‡SC: Subcutaneous route

Of these 19 experimental sera, 15 were found to be useful for the serotyping of the strains tested (Tables 14 and 15). One antiserum which was prepared against a group C strain R80/3705 did not produce a T-antibody response in the rabbit and was withdrawn from the set. This strain was later biochemically identified as S.zooepidemicus. Three further antisera were also withdrawn as they did not agglutinate with any other strain apart from their own homologous vaccine strain.

Antibody titres against the group and T antigens are illustrated in Tables 14 and 15. Initially, eight whole cell trypsinised vaccines were prepared against Lancefield group C strains. In all rabbits, antibody responses to both the group and T antigens were detected at two weeks following the primary inoculation. The agglutination titre of the group antibody varied from 1 in 1000 to 1 in 10,000 over the eight week inoculation period. After week six, the titres began to decrease. Antibody to the T protein was also apparent at week two but the agglutinin titres were slightly lower ranging from 1 in 500 to 1 in 10,000. Precipitin titres were, not unexpectedly, considerably lower ranging from 1 in 2 to 1 in 8.

Table 15 illustrates the response in 12 rabbits to different immunogens of Lancefield group G streptococci. Both the group polysaccharide and T protein antibodies were apparent as with group C, at week two. Titres to the group were from 1 in 200 to 1 in

Table 14

Antibody response to whole cell trypsinised vaccines of Lancefield group C streptococci

Strain no.	Titres	
	Group	T protein
NCTC 4540 (7T)	10,000	8000
NCTC 5370 (20T)	10,000	10,000
NCTC 5371 (21T)	10,000	8000
* R80/3705	1000	0
R80/5582	1000	600
R80/4225	1000	800
R81/1058	1000	500
R82/1925	1000	500

* Mucoid strain

Table 15

Antibody response to whole cell trypsinised vaccines of Lancefield group G streptococci

Strain no.	Titres	
	Group	T protein
NCTC 5969	10,000	8000
R80/5356	2000	1000
R80/5007	10,000	10,000
R80/4327	1000	800
* R80/6866	500	500
* R80/7023	1000	800
* R80/7118	500	500
R81/1986	200	100
R82/1094	1000	500
R82/1847	1000	800
R82/1965	1000	800
R81/5162	1000	800

* Withdrawn from the T-typing set of antisera

10,000 and titres to the T protein ranged from 1 in 100 to 1 in 10,000. Precipitation titres were similar to those obtained with the group C strains. The intravenous route of inoculation was used for each rabbit.

Absorption of the group polysaccharide antigen with standard cell suspensions

Heavy absorption procedures were always required for the removal of the group polysaccharide antibody. Standard strains of group C and group G namely, R79/3540 and NCTC 11825 respectively were used for all absorption procedures. Room temperature (R.T.) absorptions of 4-72h were performed. Usually, 48h at R.T. was adequate for most sera. The absorption procedure was shortened to 30min-1h by incubation at 37°C. The group antibody was therefore removed which permitted the detection of antibody to the T protein by slide agglutination and immunoprecipitation.

Absorption of the group polysaccharide antibody by affinity chromatography

The antigen determinant of Lancefield group A is N-acetylglucosamine and that of group C is N-acetylgalactosamine. Lectin gel preparations coupled to either one of these sugars are available commercially (Affi-gel, Bio-Rad Laboratories). The gel coupled to the N-acetylgalactosamine sugar was used in an attempt to remove group antibody from a serum prepared against a Lancefield group C strain, T-type 7.

After treatment of the serum with the Affi-gel preparation at 4°C for at least 24h, the group antibody could not be detected by slide agglutination. It could however still be detected as a weak precipitin line in gel diffusion tests. This method was found to be more time consuming, less efficient and more expensive than the use of the whole cell absorption procedure.

Specificity

Multiple reactions were occasionally found with particular strains. For example, agglutination with the sera 7 and 302 occurred only among strains of Lancefield group G. T-type 302 always occurred as a complex pattern with serotype 7 in these organisms. Strains of Lancefield group C carrying the T-type 7 antigen did not react with serotype 302 antiserum. Similarly, strains of type 301 were always agglutinated by both 301 and 305 antisera, whereas serotype 305 also occurred as a single distinct serotype and did not react with the 301 antiserum. These were the only multiple reactions detected in the initial experimental serotyping set of antisera prepared from whole cell immunogens.

The T-protein antibody response to whole cell saline vaccines

Table 16 illustrates the antibody response in rabbits to the T proteins of some representative strains of Lancefield group C and group G streptococci. For both groups, high titres of the group polysaccharide and T-protein antibody were obtained. These were usually detected by slide agglutination two weeks after the initial primary dose had been given. Titres to the T antibody were

Table 16

The antibody response to whole cell saline vaccines of Lancefield group C and group G streptococci

Group	T type	Agglutination titres to the group antibody at week			Agglutination titres to the T protein at week		
		2	4	6	2	4	6
G	7/302	100	500	*B.O.	10	500	B.O.
G	303	100	1000	B.O.	10	500	B.O.
G	†16TS	10	400	10	100	1000	800
G	‡16BC	-	200	10	50	800	400
G	300BC	-	400	10	100	400	400
G	307	2	2	5	100	10	10
G	21	2	5	5	10	100	100
G	301BC	2	5	5	10	100	100
G	305	2	2	-	2	10	10
C	7	10	10	2	10	100	10
C	21	-	-	2	-	2	5
C	204	-	500	B.O.	100	500	B.O.
C	305	10	100	500	10	100	500
C	308	10	400	10	100	400	200

*B.O. = Bled out
†TS = Throat isolate
‡BC = Blood culture

similar to those obtained for the group antibody response. The titres for the group varied according to the vaccine strain used. For the Lancefield group C streptococci the peak agglutination titres ranged from 1 in 2 to 1 in 500 and this was usually detected at week four of the inoculation programme. Similarly, titres to the T-protein antibody also varied depending on the vaccine strain. The titres ranged from 1 in 2 to 1 in 1000. An inoculation schedule as previously described over a six week period was usually required to obtain reasonable antibody titres to the T protein.

Antibody response to the T protein using whole cell trypsinised vaccines

It is evident from the data in Tables 14 and 15 that the antibody response to both the group polysaccharide and the T protein is somewhat higher using trypsinised cells. It seems likely that some antigenic material on the bacterial cells in exposed by the proteolytic enzyme. Antibody titres for the group polysaccharide varied from 1 in 200 to 1 in 10,000 and the titres for the T protein ranged from 1 in 100 to 1 in 10,000. Both antibodies could be detected by slide agglutination at two weeks following the primary dose of vaccine. However, as with antisera prepared from whole cell saline immunogens, heavy absorption procedures were required for the removal of the group antibody. The absorption procedures were required for each antiserum produced from a total of 30 inoculated rabbits.

Both slide agglutination and immunoprecipitin tests clearly

indicated the presence of antibodies to the T protein. The immunoprecipitin tests also indicated the presence of antibodies to trypsin. These did not however interfere with the specificity of the slide agglutination tests.

Antibody response to other species of Lancefield group C and animal isolates of both groups C and G streptococci

Rabbits were also immunised with trypsinised whole cell immunogens of other species of Lancefield group C namely, S.dysgalactiae, S.equi and S.zooepidemicus and also with animal isolates of Lancefield group G streptococci. Group antibody was produced in abundance with agglutination titres of up to 1 in 10,000. After absorption to remove the group antibody, slide agglutination tests with even the immunising strains were all negative (Table 17). The rabbits were subsequently boosted with further doses of vaccine with the same result. It was concluded that T-protein antigens may not be present on representatives of other species of Lancefield group C and from animal strains of Lancefield group G.

3.2.3. PRODUCTION OF ANTISERA TO THE T PROTEIN USING PARTIALLY-PURIFIED, ISOLATED PROTEINS

The isolation of the T protein was based on the original method described by Pakula (1951). There have been several reports on the isolation of T protein from Lancefield group A streptococci, for special absorptions of antisera (Pakula 1951, McLean 1953, Erwa 1973). It was decided therefore, to explore the use of these methods to isolate a T-protein preparation, devoid of group

Table 17

Antibody response in rabbits to trypsinised immunogens of Lancefield group C and group G streptococci

Species	Lancefield group	Vaccine strain no.	Source	Group antibody	T antibody
S.equisimilis	C	*See Table 4	Human	+	+
S.dysgalactiae	C	NCTC 4669	Cow	+	-
"	"	NCTC 4335	Cow	+	-
"	"	NCTC 4671	Cow	+	-
S.equi	C	NCTC 9682	Animal	+	-
S.zooepidemicus	C	NCTC 6176	Mouse	+	-
Streptococcus sp.	G	*See Table 4	Human	+	+
Streptococcus sp.	G	NCTC 7932	Dog	+	-

* Vaccine strains used in the production of T-typing antisera for the serotyping scheme.

polysaccharide, for use as an immunogen in the production of T antisera.

Several inoculation protocols were used but with no striking difference in results between the different routes of inoculation. Subcutaneous and intravenous routes with and without the presence of an adjuvant (aluminium hydroxide) were explored in a total of 33 rabbits. T-protein extracts were prepared for all T-types of Lancefield groups C and G (Table 4). Rabbits were inoculated with 200-400 µg/ml of extracted protein. They were injected over a six week period and bled at weekly intervals. The test bleeds for each type antigen were studied individually for the presence of group specific and T-protein antibodies by slide agglutination and immunoprecipitation. The immunoprecipitin tests clearly indicated the presence of T protein and also the presence of trypsin contamination in the extract (results not shown).

The different protocols of inoculation in the rabbits did not lead to any differences in antibody responses (Fig. 2 and Table 18). It was therefore decided to administer the vaccines without the presence of an adjuvant and by the intravenous route of inoculation. Crude trypsin (Difco 1:250) was the extraction enzyme used.

Antibody titres to the isolated T protein

The antisera were tested for precipitin activity with the isolated T antigens by double gel diffusion and yielded titres of 1 in 2 or

Table 18

Antibody response to partially purified T proteins of Lancefield groups C and G streptococci

*Strain	†T antibody titre			Emergence of group antibody at week
300	4	‡at week	3	4
301	8	"	4	4
302	8	"	4	4
303	8	"	5	3
304	4	"	4	4
305	16	"	5	5
306	8	"	4	4
307	8	"	4	4
308	16	"	5	5
200	4	"	3	4
201	8	"	4	4
202	4	"	5	4
203	8	"	4	4
204	16	"	5	4

* vaccine stock strains
† agglutination titre
‡ week during inoculation schedule when test bleed was taken

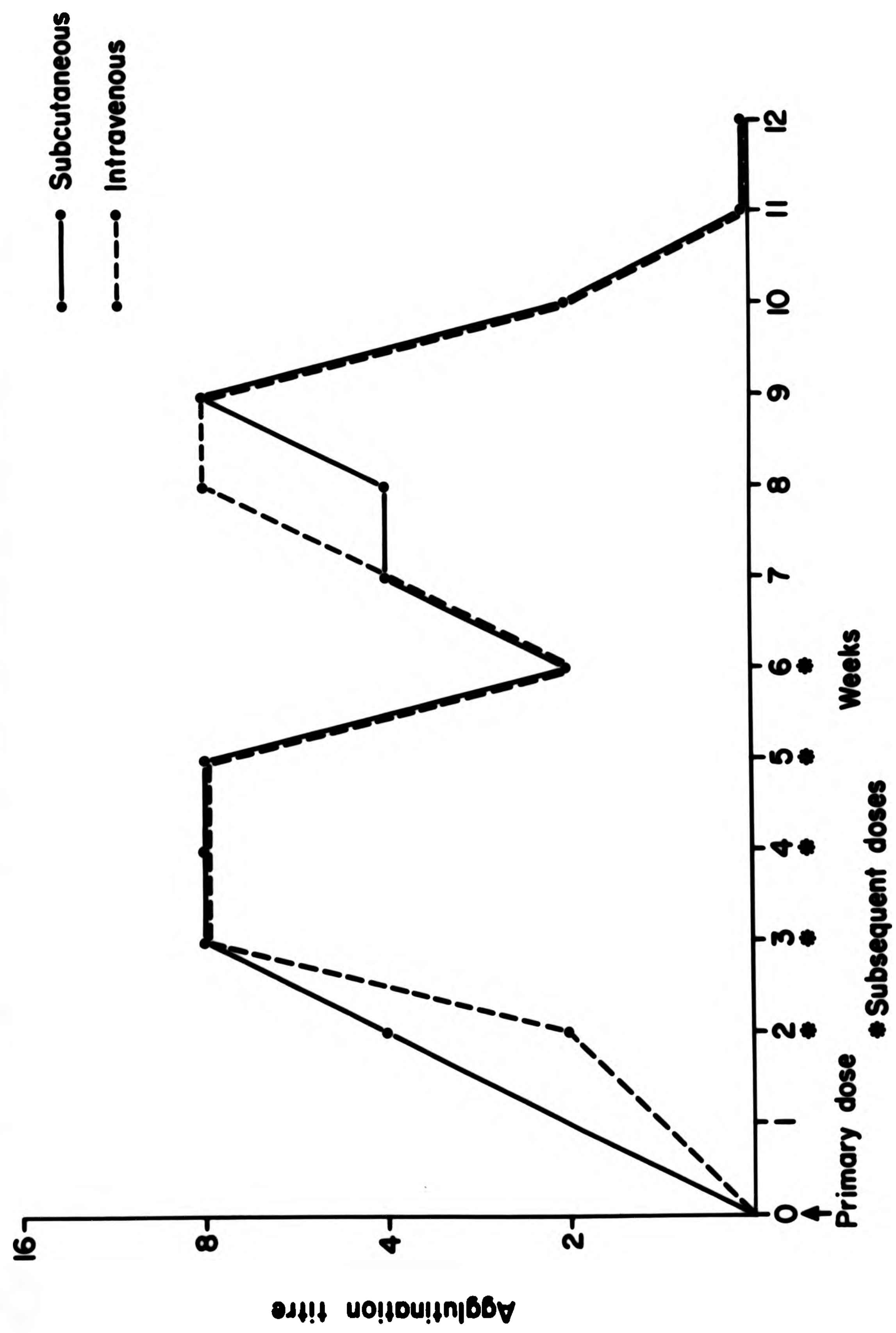


Fig. 2 Antibody response to T-protein extracts using different routes of inoculation

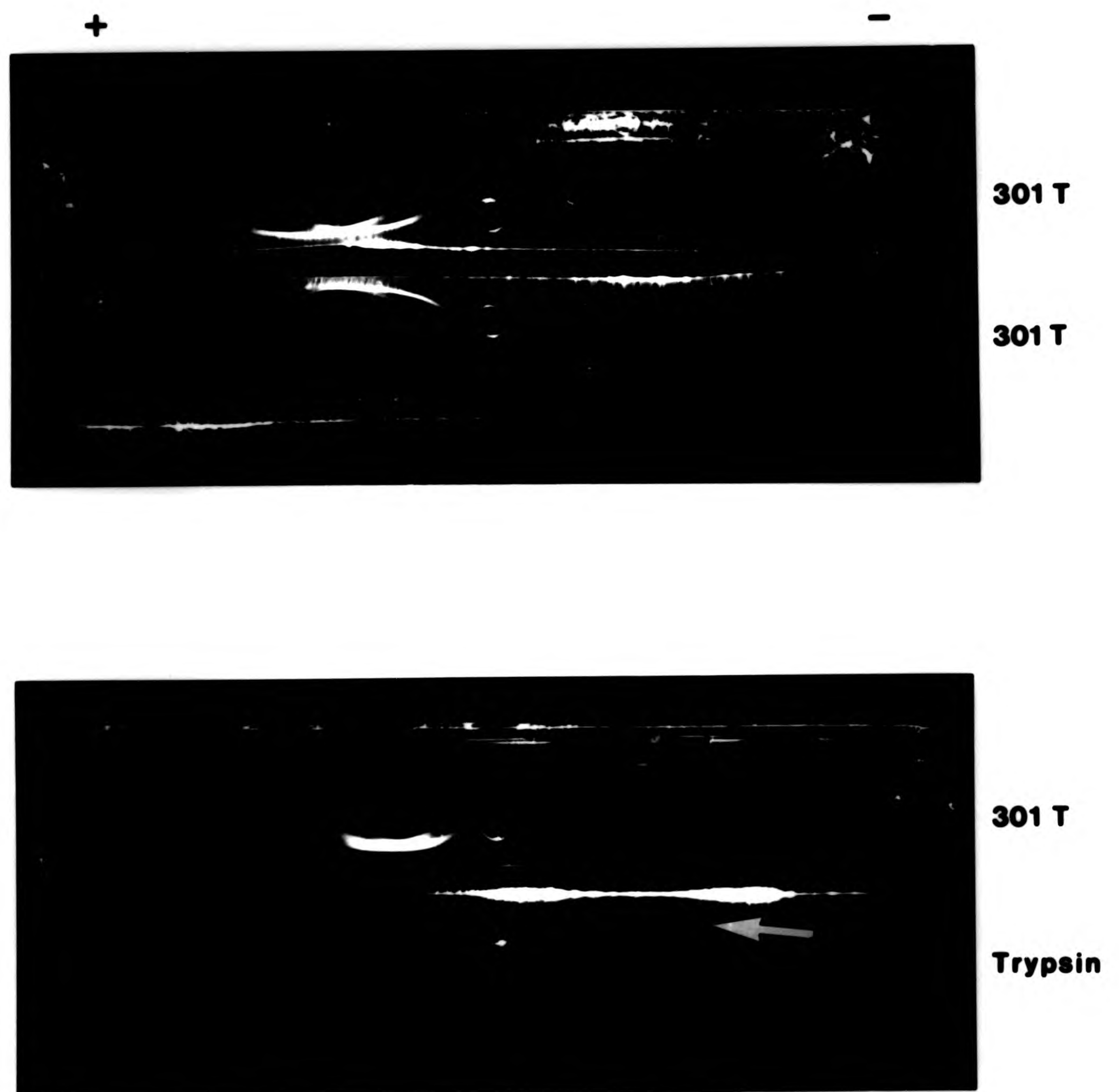
1 in 4. Agglutination titres obtained with the isolated T-protein extract were fairly consistent for each strain. The T antibody began to emerge at week two after the first dose of antigen. The titres were lower than those obtained with whole cell immunogens, being between 1 in 4 and 1 in 16 (Table 18). Rocket immunoelectrophoresis was also used as an additional method to determine the precipitin activity of the T protein, but was not very valuable as a constant monitoring technique for T-protein activity.

Antibodies to the group polysaccharide could be detected during the fourth and subsequent weeks of immunisation. Antibodies to trypsin could be demonstrated in some undiluted sera by gel diffusion and immunoelectrophoresis (Fig. 3). As sera were usually diluted 1 in 4 for use in the slide agglutination tests there was no apparent interference with the T-typing tests. Immunodiffusion of the isolated T extract against its homologous antiserum clearly demonstrated the presence of trypsin as a precipitin line of partial identity with the T protein. Countercurrent immunoelectrophoresis also indicated the presence of trypsin in the T-protein extract (Fig. 3).

<u>Specificity of the antisera prepared from isolated T-protein extracts</u>

No differences were found in the specificity of the antibodies irrespective of whether a whole cell immunogen or an extracted antigen was used. Multiple reactions were occasionally found with

Fig.3 Countercurrent immunoelectrophoresis of serotype 301T to demonstrate T-protein and trypsin activity



Antibody: serotype 301 prepared from a trypsinised vaccine.

particular strains, as noted earlier (see Section 3.2.2.). There was no evidence that these reactions were more, or less, frequent with the trypsin extracted T protein (isolated antigen) than the whole cell immunogens.

T-protein immunogens extracted with different enzyme preparations

Generally, trypsin 1:250 (Difco) was used for all extraction procedures. It was decided to explore the use of a purified trypsin preparation as a comparison. Crystalline trypsin (from bovine pancreas, type XII, 2 x crystallised, salt free: Sigma Chemical Co.) with a stated enzymatic activity of 750 BAEE units/mg protein (based on $E^{1\%}_{280}$ = 14.4, one BAEE unit = Δ A253 of 0.01 per minute in 3.2 ml at pH 7.6 at 25°C), was used. Trypsin preparations with proteolytic titres of 10^{-3} or greater on exposed and developed photographic film and active in immunodiffusion were used for all extraction procedures. Crystalline trypsin fulfilled these specifications at a final concentration of 0.1 mg/ml.

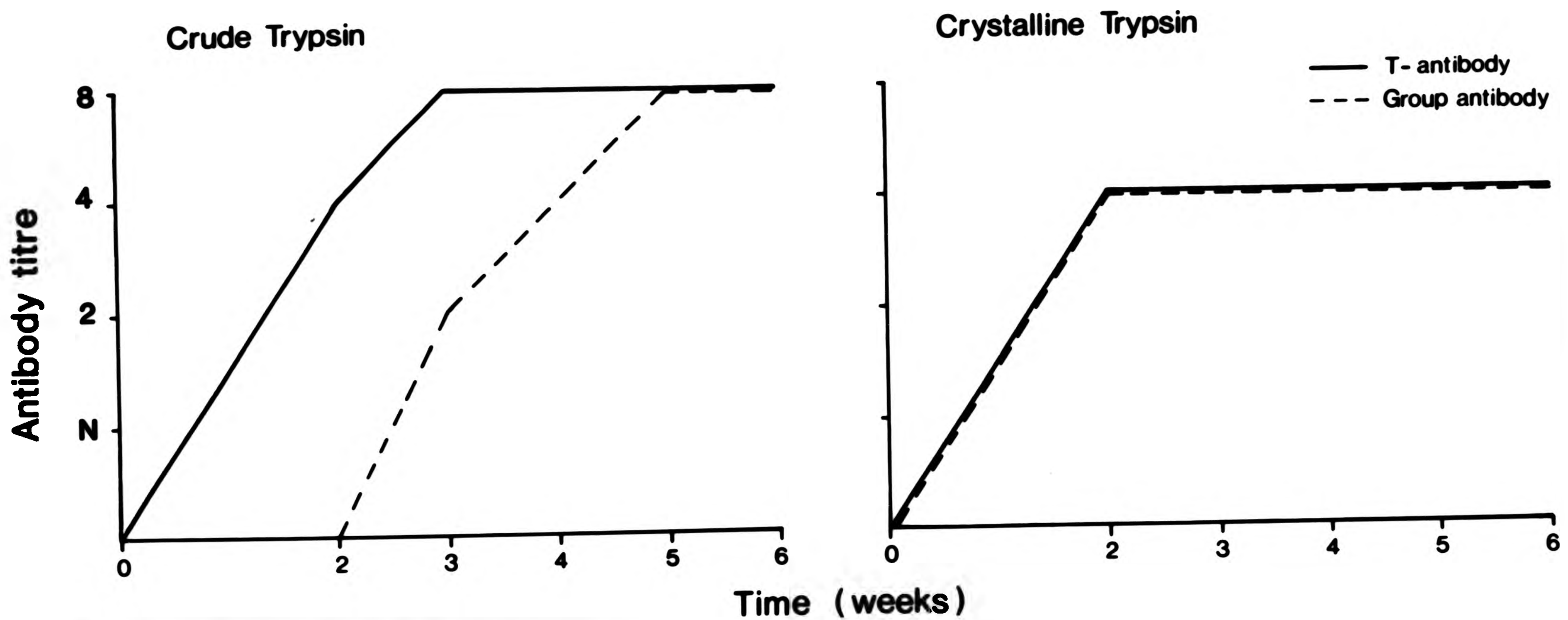
Two random strains were selected for this experiment, a group G with the T antigen 301 and group C serotype 203. T antigens were isolated and extracted from each strain using the crude and purified enzyme preparations. The protein content for each extract was determined and this varied from 0.2 - 1 mg/ml. The presence of trypsin could be demonstrated on photographic film where the titres were 10^{-1} or 10^{-2}. Antigens extracted by the crystalline enzyme produced a lower yield of antigen (0.2 - 1 mg/ml), than the extract prepared using the crude enzyme (0.4 - 2

mg/ml). Fig. 4 demonstrates the difference in antibody response using the two enzyme preparations. The group polysaccharide emerged at an early stage (week 2) with antigens extracted using crystalline trypsin. The group antibody response paralleled that of the T antibody. T-antibody titres were found to be slightly higher with antigens extracted by the crude enzyme (8 as opposed to a titre of 4). The highest titre achieved with the purified enzyme extraction was 1 in 4. There was therefore no apparent advantage in using the purified enzyme rather than the crude material.

Antibody response to the filtered and non-filtered T protein immunogens

Initial studies employing a strain of Lancefield group C, type 204 and one of group G, type 308, were evaluated. The isolated T-protein preparations were injected into individual rabbits and the antibody response monitored during a six week immunisation schedule. The T-antibody titres obtained with both filtered and non-filtered immunogens were very similar (Fig.5). As expected antibody to the group polysaccharide began to emerge during the fourth week with the unfiltered preparation. Antibody to the group polysaccharide was not detected in animals that had been injected with the filtered vaccines. The T antibody could be detected in both agglutination and precipitation tests. Table 19 illustrates the titres obtained with the preparations derived from serotypes 204 and 308 using filtered and unfiltered material. Antibodies to trypsin could be detected only in undiluted antisera

Fig. 4 Comparison of the antibody response to streptococcal T-protein extracted with a crude and pure enzyme preparation



N : agglutination with neat antiserum

Fig. 5 Comparison of the antibody response in rabbits to the T-protein using filtered and non-filtered immunogens. ■—■ = T antibody response; - - - - = group antibody response.

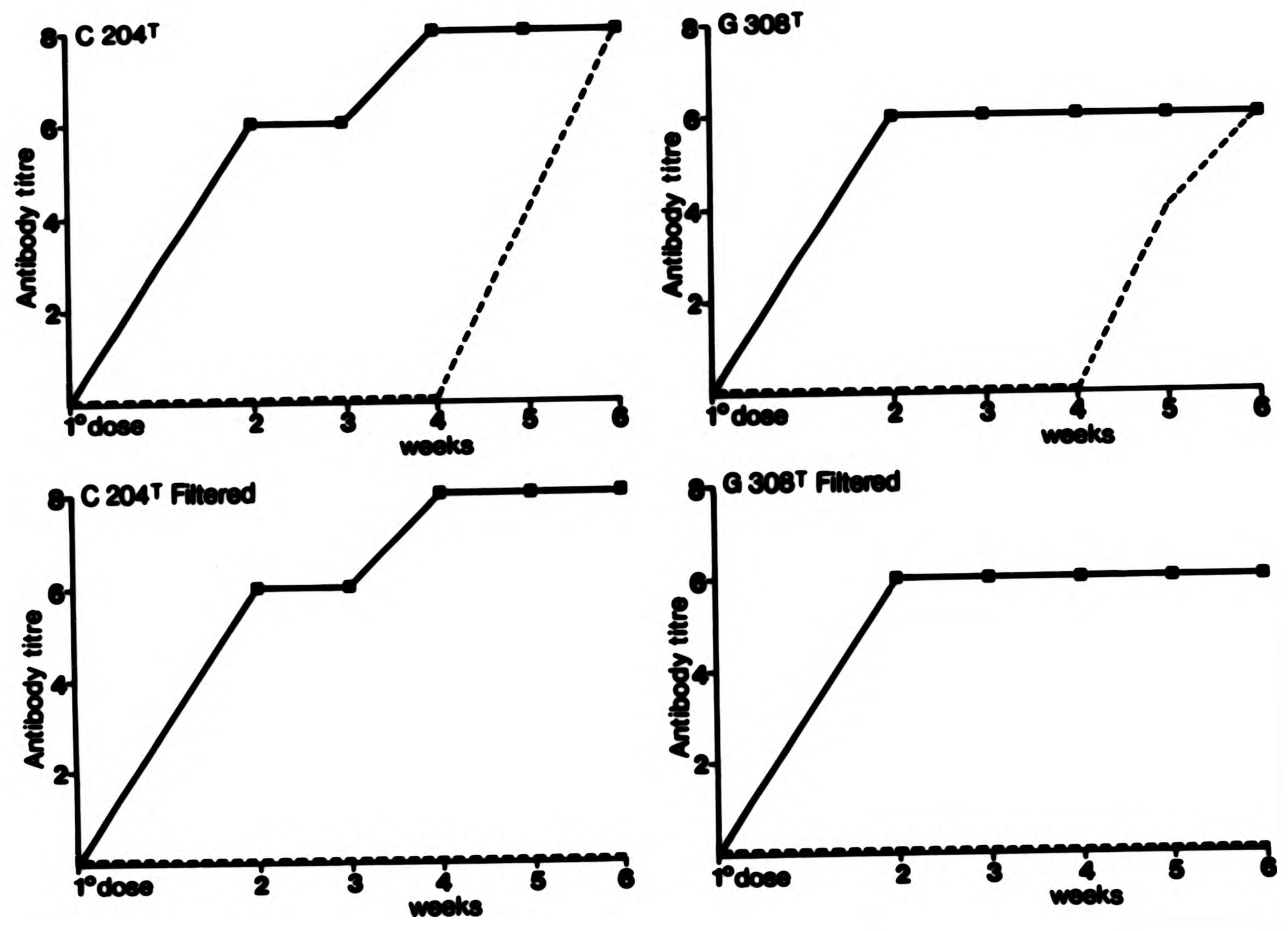


Table 19

The production of antisera to T proteins using non-filtered and filtered immunogens

Immunogen	Antibody Response					
	T		Group		Trypsin	
	*aggln	†pptn	aggln	pptn	aggln	pptn
Type 204 (non-filtered)	8	2	8	2	weak	0
Type 204 (filtered)	8	2	0	0	weak	0
Type 308 (non-filtered)	6	NEAT	6	2	0	0
Type 308 (filtered)	6	NEAT	0	0	0	0

* aggln = agglutination titre

† pptn = precipitin titre

The protein content for each immunising dose was equivalent to 400µg/ml.

during the fourth week of immunisation. Representatives of all T types were subsequently examined using filtered vaccines as immunogens. The results obtained were consistent for both groups and identical to those obtained in the preliminary study. The antisera prepared from these isolated T proteins were always type specific. They did not cross-react with strains belonging to other Lancefield groups; in particular those of group A.

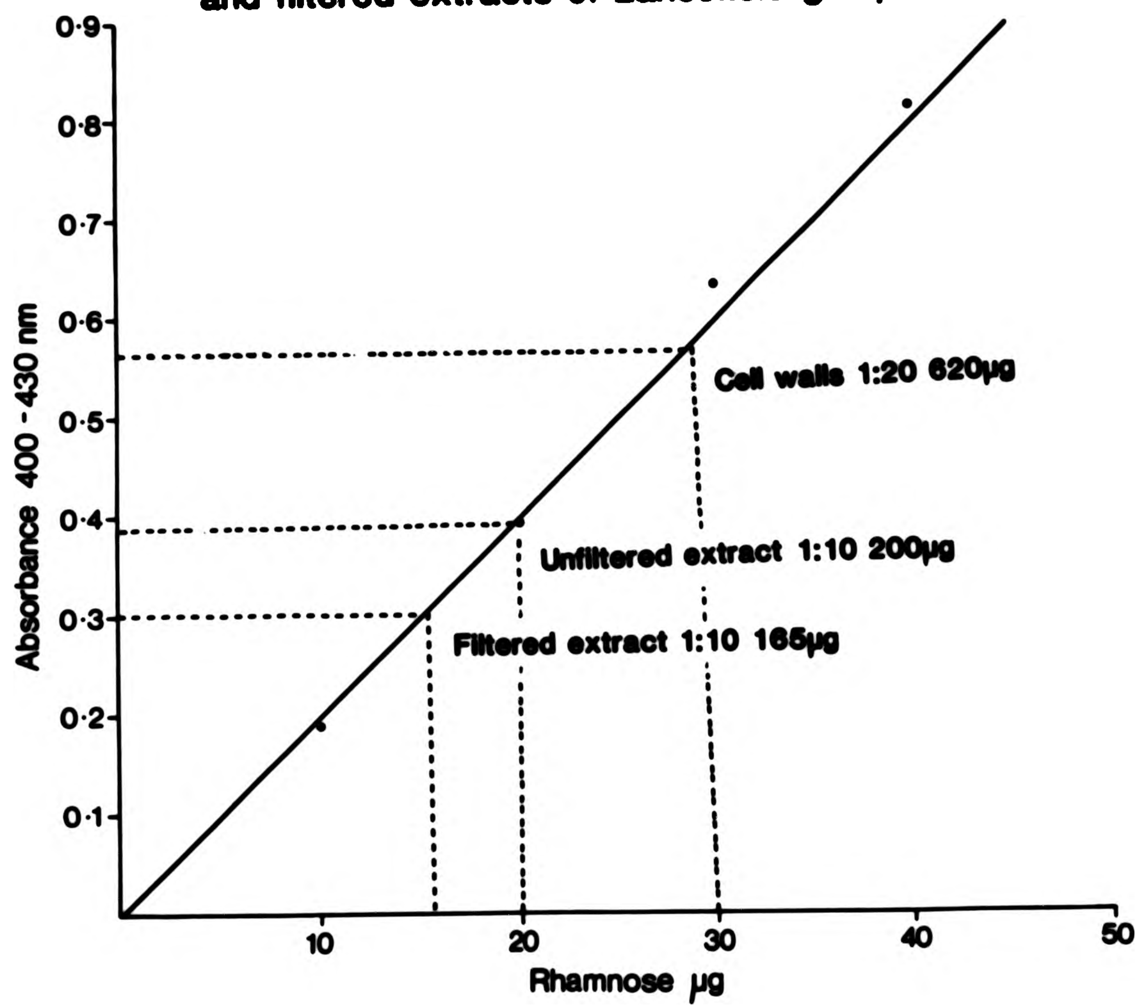
Rhamnose is characteristically found in the cell walls of pyogenic streptococci and moreover is the determinant sugar of the Lancefield group G antigen. Thus, the amount of rhamnose (per mg dry weight) was compared for whole cell walls on the one hand and the unfiltered and filtered supernates on the other. A strain of Lancefield group G serotype 308 (NCTC 11630) was used. The rhamnose content was found to be 1.0 and 0.8% of the dry weights of the unfiltered and filtered extracts respectively, and 12% of the dry weight of whole cell walls (see Appendix 2 and Fig.6).

<u>Electron microscopy</u>

The cell walls and the T-protein preparations (unfiltered and filtered extracts) were examined by transmission electron microscopy to determine the presence of any cell wall debris in the unfiltered extract which could perhaps be associated with the stimulation of the antibody response to the group antigen.

In the negatively stained preparations the whole cell walls were visualised as collapsed structures without adhering debris (Fig.

Fig. 6 Determination of the rhamnose content in the cell walls, unfiltered and filtered extracts of Lancefield group G streptococci



7). Bacterial cells and cell walls were not found in the T-protein extracts, but granular material lacking a recognisable structure was seen in the unfiltered material (Fig.7). This material was not present in the filtered supernate.

The digestion and extraction of T proteins with other proteolytic enzymes

Peptic extracts of different T proteins were prepared and their antibody response in rabbits was monitored over a 16 week inoculation period. Figs. 8 and 9 demonstrate the antibody response to the T-protein antigens of six representative strains of Lancefield group C and six representatives of Lancefield group G. Group polysaccharide was not detected. The average T-antibody titre was 1 in 16. Only two out of the twelve strains examined produced T-antibody titres of less than 1 in 2. The peak antibody response was produced during weeks 4 to 8 of the inoculation schedule. Cross reactivity between strains of different T types was less apparent using antisera that had been prepared from pepsinised immunogens. Hence, type specificity was greater.

Table 20 summarises the differences in the antibody response using the different preparations. Overall, the agglutination titres obtained with the isolated T extracts were lower than the titres obtained with whole cell vaccines. The antisera prepared however, from whole cells contained higher titres of the group polysaccharide antibody, usually within the range of 1 in 5,000 to 1 in 10,000. Therefore, for removal of the group antibody the

Fig.7 Electron micrographs of a) cell debris present in non-filtered immunogens and b) streptococcal cell walls of Lancefield group G

a)

b)

Print magnification a)x75,000 b)x50,000

Microscopic magnification a)x30,000 b)x20,000

Fig. 8 The T-protein antibody response to peptic extracts of Lancefield group C streptococci

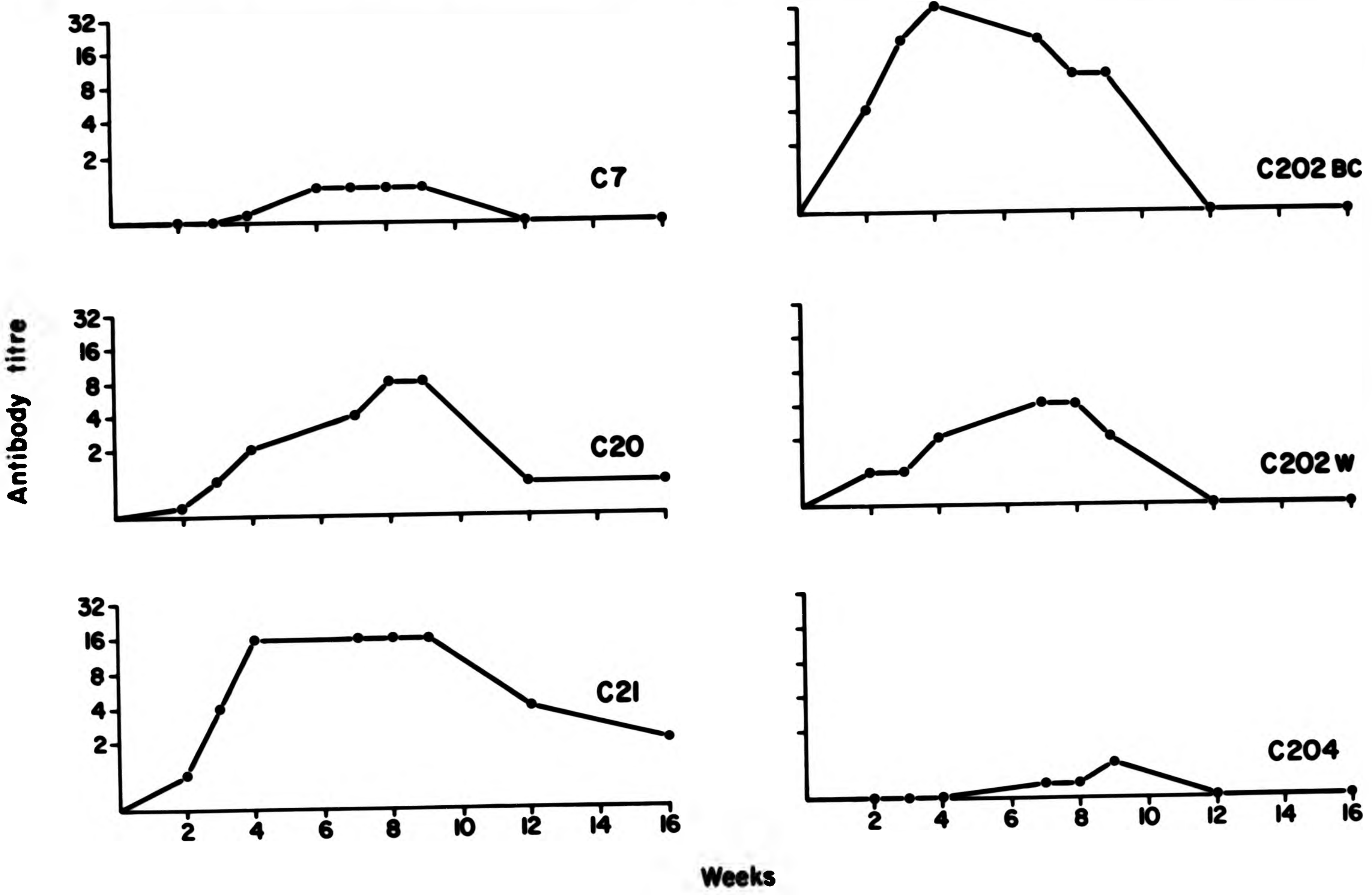


Fig. 9 The T-protein antibody response to peptic extracts of Lancefield group G streptococci

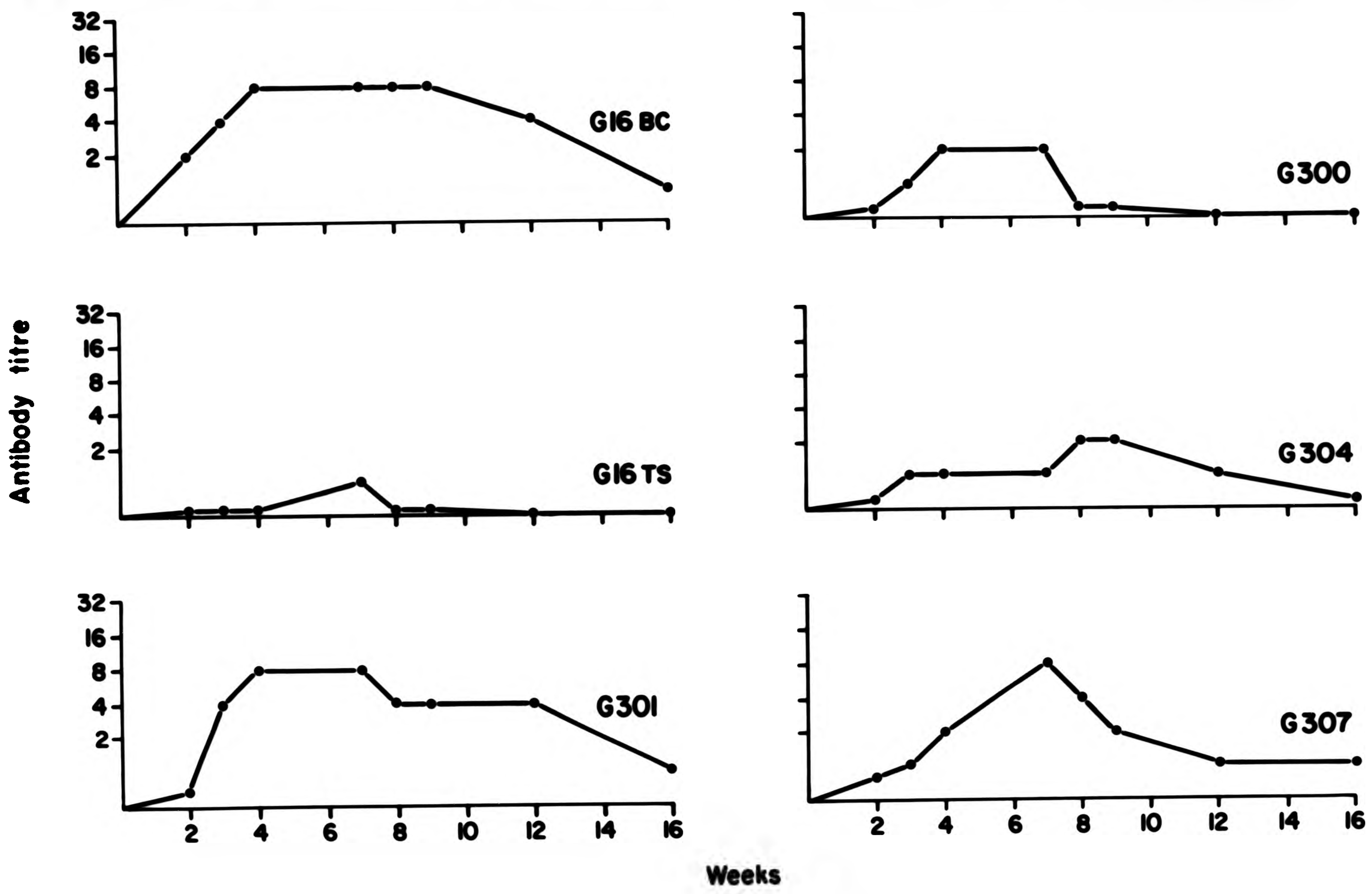


Table 20

The use of different vaccines in the production of antisera to the T protein

Vaccine	Antibody Response			Absorption
	T	Group	Trypsin	
Whole cell saline	+	+	+	Required
Whole cell trypsinised	+	+	+	Required
Unfiltered T extract	+	+/-	±	Sometimes required
Filtered T extract	+	-	±/-	Not required
Pepsin extract	+	-	-	Not required

antisera required heavy absorptions with packed streptococcal cells over a period of 48h. The T-antibody titre from the absorbed antisera varied from 1 in 500 to 1 in 5000. It was not possible to determine the titre before absorption as this was masked by the group antibody.

Antisera prepared from isolated T proteins were generally type specific with titres of approximately 1 in 8. Production of the group polysaccharide antibody could be avoided using the filtered T extracts as vaccines.

The use of pepsin extracted immunogens induced an antibody response to the T protein but as with antisera prepared from isolated T proteins, the antibody titre although specific was somewhat low.

Cross-absorption of antisera to determine specificity

The presence of heterologous reactions between various strains was observed during the course of the study. Attempts were made to remove these additional reactions using isolated T-proteins. Reactions with more than one T-type were rarely encountered with the Lancefield group C streptococci but were seen more frequently with the Lancefield group G streptococci.

All group G streptococci with the T antigen 302 share an additional antigen with type 7, whereas type 7 may occur alone with both groups. A T-antigen precipitate of serotype 302 (vaccine strain) was prepared and type 7 antiserum was absorbed with this precipitate. The typing results obtained are shown in Table 15, before and after absorption of the antisera. Absorption of

antisera with cross-reacting strains belonging to a different group (for example, strains of group G with either the T-type 7, 20 or 21) demonstrated that these were genuine T-type reactions common to both groups C and G (Table 21). Thus an antiserum prepared against a group C serotype 20 strain when absorbed with cells of a group G serotype 20 strain will lose its T-protein antibody activity.

Other complex patterns encountered were with strains of Lancefield group G reacting with both types 301 and 308 antisera, and also strains reacting with serotype 301 and 305 sera. These cross-reactions were not encountered with antisera produced from pepsinised vaccines. It would appear that pepsin extraction either did not liberate the second T-protein antigen or destroyed that epitope (Table 22).

Crossed immunoelectrophoresis experiments were performed to determine the presence of two distinct T proteins in strains of Lancefield group G which exhibited cross reactivity with the T antisera 301 and 308. Two distinct peaks were recognised (Fig.10). These peaks could be the result of antibodies reacting with:-

i) T protein and group antigen

ii) T protein and trypsin

iii) Two distinct T proteins

The first possibility could be excluded by the absence of group antibody as detected by either slide agglutination or gel diffusion

Table 21

Agglutination reactions of T antigens before and after cross absorption

I. Before Cross-Absorption

Strains	Antisera			
T type	C7	G302	C20	C21
G 7	+	-	-	-
G 7/302	+	+	-	-
C 7	+	-	-	-
C 20	-	-	+	-
G 20	-	-	+	-
C 21	-	-	-	+
G 21	-	-	-	+

II. After Cross-Absorption

Strains	C7abs*	G302	C20abs†	C21abs‡
G 7	+	-	-	-
G 7/302	-	+	-	-
C 7	+	-	-	-
C 20	-	-	-	-
G 20	-	-	-	-
C 21	-	-	-	-
G 21	-	-	-	-

* 7abs: type 7 antiserum absorbed with the T extract of type 302

† 20abs: type 20 antiserum absorbed with T extract group G type 20

‡ 21abs: type 21 antiserum absorbed with T extract group G type 21

Fig. 10 Crossed-immunoelectrophoresis of the T-protein extract serotype 301 (Lancefield group G)

Antigen: 301T

Antibody:Serum prepared from a trypsinised vaccine of serotype 301

Table 22

Effect of proteolytic enzymes on the release of T proteins and antibody production

T type	Antisera prepared from:					
	Trypsinised extracts			Pepsinised extracts		
	301	305	308	301	305	308
301	+	+	+	+	-	-
305	+	+	-	-	+	-
308	-	-	+	-	-	+

+/-: Agglutination reaction with neat antiserum and trypsinised streptococcal suspension

Table 23

Results of crossed immunoelectrophoresis (CIE) on group G streptococci with the T-complex pattern 301/308

T extract	Agglutination reaction with :-		No. of CIE peaks produced in gel containing antibody against:-	
	301	308	301	308
301T	+	+	2	2
308T	-	+	0	1
*301T	+	±	2	not done

* Trypsin free extract (trypsin was separated from the T protein by affinity gel chromatography, see Section 3.3.4.)

tests. The second possibility may also be excluded as a 'trypsin-free' preparation of serotype 301 also produced two peaks identical to those produced by a T extract containing trypsin. The third possibility appeared to be the most likely with two distinct T proteins being liberated in trypsin extracted preparations. The results of crossed immunoelectrophoresis on the T extracts of serotypes 301 and 308 are demonstrated in Table 23.

Summary

Antibodies to the T protein were effectively induced in rabbits. The T - protein antigens of Lancefield group C and group G streptococci were found to be excellent immunogens. The titres of agglutinating antisera prepared from whole cell vaccine preparations ranged from 1 in 500 to 1 in 10,000. In parallel with this there were also high titres of group specific antibody, thus heavy absorption procedures were required for removal of the antibody. The use of trypsin extracted T proteins as immunogens largely avoided the need for these absorptions. The trypsinised cell walls of Lancefield groups A, C and G streptococci contain two major components, the mucopeptide matrix consisting of N-acetylmuramic acid, N-acetylglucosamine and amino acids; and the group specific carbohydrate which for Lancefield group G is mainly composed of rhamnose. Rhamnose was detected in the T-protein extract preparations of Lancefield group G strains at lower levels than in the intact cell walls.

Group antibody also became apparent in the isolated T-protein

extracts but was effectively overcome by filtration of the extract prior to acid precipitation of the protein material. This was demonstrated by electron microscopy by the presence of particulate matter in the unfiltered extract and the group antibody response in rabbits was associated with this material. Pepsin extracted streptococcal digests were also effective in the stimulation of antibodies to the T protein. Although the T-protein antibody response to the partially purified material was of a lower titre in comparison to a whole-cell vaccine, the response itself was type specific.

3.3. CHARACTERISATION OF THE T PROTEIN

3.3.1. EXTRACTION AND ISOLATION OF THE T PROTEIN

As T proteins are resistant to degradation by proteolytic enzymes such as pepsin and trypsin, this property has therefore provided a basis for their extraction from streptococci (Lancefield and Dole 1946). The T-protein antigens of Lancefield group C and group G streptococci were extracted by a modification of the methods of Pakula (1951) and McLean (1953). The average protein yields for the T-extract preparations varied between 1-4 mg/ml of crude protein material from 8l of original broth culture. The T-protein activity of the extract was determined by immunodiffusion against the homologous T antiserum. The activity of each protein extract was monitored between each purification procedure by double gel

diffusion and immunoelectrophoresis. The homogeneity of each preparation was assessed by column chromatography using gel filtration, ion exchange and affinity chromatography methods.

Isolation of the T protein by trypsin digestion and ammonium sulphate fractionation

The effect of using different trypsin preparations prior to fractionation in the isolation of the T protein was studied. The two preparations of trypsin used for the digestion procedure were the crude trypsin preparation (1:250, Difco) and the crystalline purified form (Koch Light).

T extracts prepared from representatives of each of the two groups C and G were fractionated with ammonium sulphate in an attempt to separate the T protein from the group polysaccharide (if present) and perhaps from the extraction enzyme, trypsin (Table 24). The T-precipitin activity was detected for each fraction in double gel diffusion against the homologous T antiserum. Because of the higher density of the $(NH_4)_2SO_4$ at a saturation concentration of greater than 80%, it was not possible to precipitate the T protein. However, sufficient $(NH_4)_2 SO_4$ crystals were added to obtain the 100% saturation limit and this was left overnight at 4°C. The results are illustrated in Table 24. There was greater precipitation using the crude trypsin preparation (Difco) as the extraction enzyme. The higher protein yield was also confirmed by the T-precipitin activity for each fraction (Table 24). Trypsin was present in all fractions, however the group polysaccharide

Table 24

Isolation and purification of streptococcal T protein by ammonium sulphate fractionation

Extraction enzyme	Protein content (mg/ml)	T-precipitin activity
Difco trypsin		
Precipitate (ppte) obtained from 20% saturation	0.02	-
Ppte from 40%	0.3	±
Ppte from 80%	0.5	+
Crystalline trypsin		
Ppte from 20%	0.01	-
Ppte from 40%	0.4	+
Ppte from 80%	0.2	-

could not be detected. The original protein content of the T extract before fractionation varied from 1-2 mg/ml. Therefore, it would appear that a considerable amount of protein was lost by fractionation, as the yield from the fractionation procedure was less than 1mg/ml.

Isolation of the T protein by batch adsorption

The digestion of streptococcal cells with polyacrylamide linked insoluble trypsin could produce a T-protein extract without contaminating enzyme (Table 25). Batch adsorption was used on two representative strains from each group. These were Lancefield group C, serotype strains 200 and 203, and Lancefield group G, serotype strains, 301 and 308. The washed streptococcal cells were digested with the prepared gel suspension and the digestion procedure was monitored over 24h. Samples were removed at 4h, 7h and 24h for the analysis of trypsin and T-protein activity by double gel diffusion. The samples from each strain were concentrated by ethanol precipitation or polyethylene glycol 20,000 and freeze-drying (Table 25). The samples were divided into three main categories as follows:-

A: 4, 7, 24h samples = Extracts from digestion procedure.

B: 4, 7, 24h = Ethanol concentrates of above.

C: 4, 7, 24h = Polyethylene glycol concentrates.

Trypsin was not detected in any of the samples. Consistent results were obtained for all the strains tested. Table 25 demonstrates that the polyethylene glycol concentrate from the 24h digest produced precipitation in gel diffusion with the homologous

Table 25

T-protein activity in digests of streptococcal cells treated with trypsin linked to polyacrylamide

Samples	Time (h)	T-precipitin activity
A T-protein digests	4	- [+]
	7	- [+]
	24	- [+]
B Ethanol concentrates of A	4	- [-]
	7	- [-]
	24	- [-]
C Polyethylene glycol concentrates of A	4	- [+]
	7	- [+]
	24	+ [+]

[]: denotes T-precipitin activity after further concentration of sample A, B and C by freeze-drying.

T antiserum. Activity was not detected in the unconcentrated digests and the ethanol concentrates. All samples were subsequently freeze-dried to concentrate further the T-protein material if present. The results in Table 25 indicate the T-protein activity obtained after freeze-drying. T-precipitin activity was present after 4h digestion of the cells and remained immunologically active up to 24h. However, if the digests were treated with ethanol, activity was diminished. The use of polyethylene glycol 20,000 alone for the concentration of the digest was not sufficient for the 4 and 7h samples.

When the T-protein positive samples were tested by gel diffusion along with trypsin, double precipitin lines could be detected, with the inner line common to all the samples and trypsin. The outer line of identity was common only to the T-protein positive digests. Therefore, the T-protein samples did indeed contain trypsin. This contamination could have been due to another component of the trypsin molecule that was antigenic but did not exhibit proteolytic properties, as the digests were not active on exposed photographic film.

3.3.2. PURIFICATION OF THE T PROTEIN BY GEL FILTRATION

The initial purification procedure used was gel filtration chromatography, on Sephadex G-75. Two separate strains of Lancefield group G streptococci with the T-type antigens 301 and 308 respectively, were used for all the characterisation experiments. These were the stock vaccine strains for each

serotype. After obtaining the initial crude protein yield from 8l of broth culture (2-4 mg/ml), the T-precipitin activity of the protein was determined against the homologous T antiserum (usually, unabsorbed serum which contained antibodies to both the group polysaccharide and T protein).

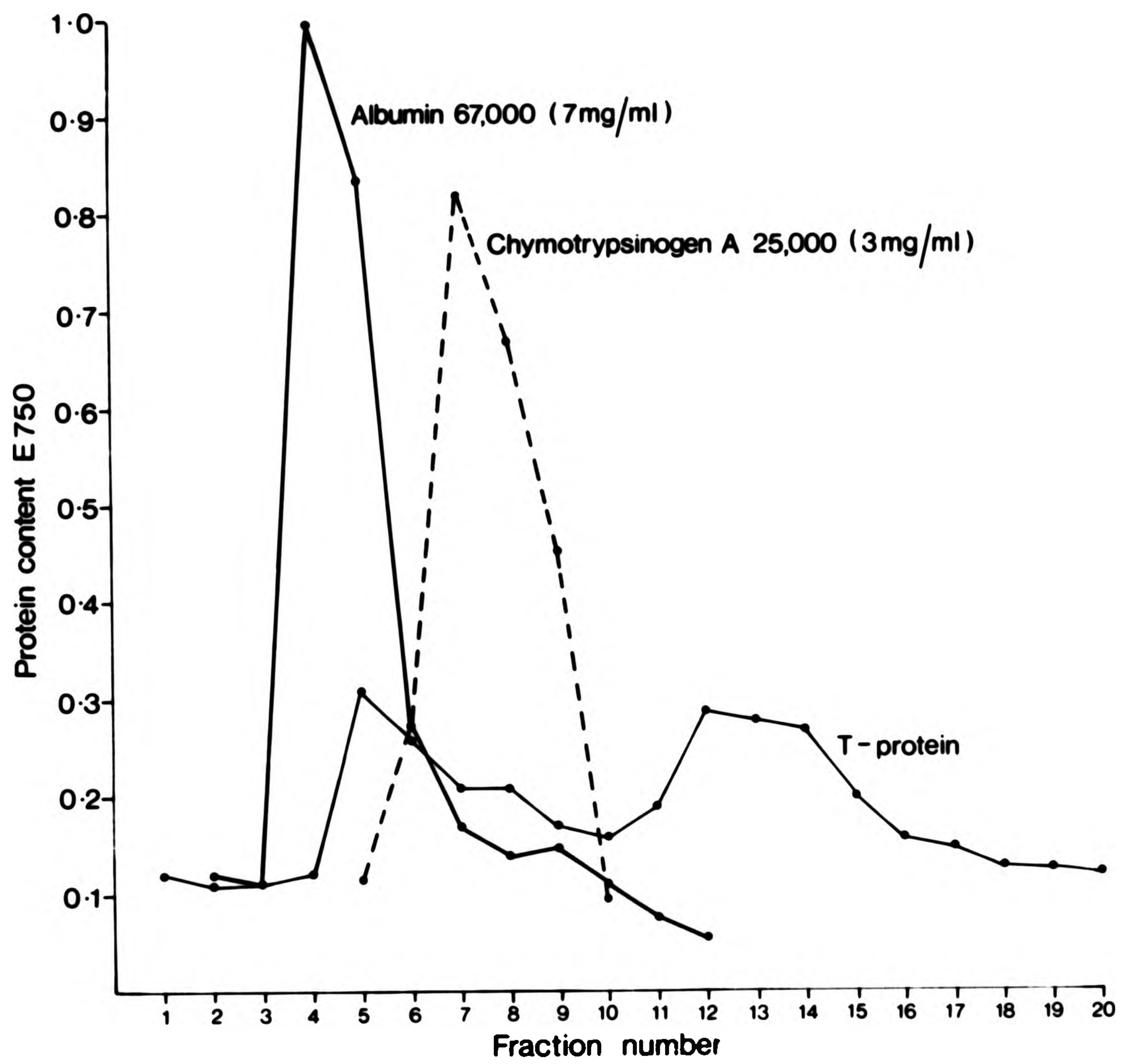
As there had not been any reports in the literature on the biochemical characterisation of the T-protein antigens of group C and group G streptococci, an initial study was made based on the separation of the crude protein material on Sephadex G-75 in comparison with the elution profiles of proteins of known molecular weights.

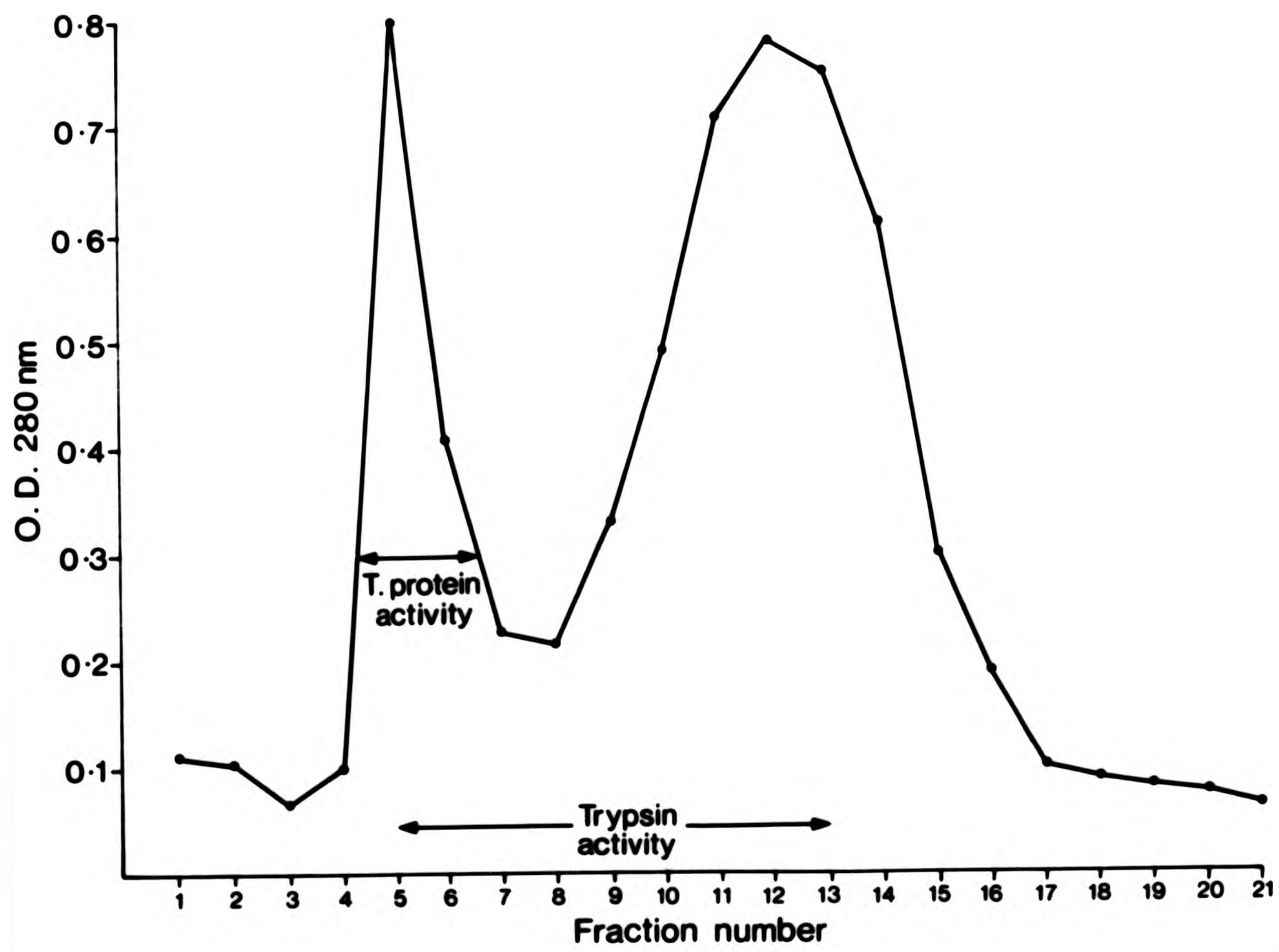
<u>Determination of the elution profiles for low molecular weight proteins and T protein by gel filtration on Sephadex G-75 and G-100</u>

The homogeneity of the T-protein extract was determined initially by gel filtration on Sephadex G-75 equilibriated with 0.01M phosphate buffer at pH 7.6. The void volume of the column was 12ml. A calibration curve (See Appendix 2) was plotted for the molecular weight markers, and fractions of 2.5ml were collected and analysed.

Separation of the T-protein extract by gel filtration on Sephadex G-75 revealed two distinct peaks (Fig. 11). The first peak could have been indicative of T-protein activity, as fractions 5 and 6 produced sharp precipitin lines in gel diffusion when tested against the homologous T antibody. The presence of trypsin was

Fig. 11 Separation of T protein by gel filtration on Sephadex G-75





determined by the proteolytic activity of the fractions on exposed photographic film. Trypsin could be detected in fractions 5 to 13. It was interesting to note the presence of two peaks for the separation of the T-protein material on Sephadex G-75. This may have been due to contamination with the extraction enzyme, trypsin. The second peak could be the product of self degradation by the trypsin itself.

The molecular weight of the T protein appeared to be nearer that of the 25,000 daltons standard, chymotrypsinogen (App. 2). However, it was still not possible to separate the T protein from trypsin by gel filtration. The protein content of the T-positive fractions 5 and 6 were 35 and 25 µg/ml respectively. Therefore, a large proportion (approximately 70-80%) of protein material had been lost by gel filtration. Similar results were obtained with Sephadex G-100.

3.3.3. ION EXCHANGE CHROMATOGRAPHY OF T PROTEIN

The separation of T protein from trypsin on the basis of its ionic properties was evaluated with the use of ion exchange resins. Both weak and strong anionic exchangers were prepared and a streptococcal T extract from the Lancefield group G vaccine strain for serotype 301 was examined.

Separation of the crude T extract on a weak anionic exchanger DEAE A-50

The crude T extract (protein content 2 mg/ml) was applied to the

column and eluted with a fairly steep salt gradient of 2M NaCl with 0.01M sodium phosphate buffer, pH 7.6 as the eluent. The T-precipitin activity prior to chromatography was previously determined in gel diffusion. The absorbance readings for each fraction were taken at 280nm and a graph was plotted accordingly (Fig.12). The graph revealed a sharp peak between fractions 12 and 18. Protein determinations were performed on each fraction (Fig. 12) by the method of Lowry with a series of albumin standards. The T-positive fractions as determined by gel diffusion produced a protein yield of 25-40 µg/ml. T-protein activity was detected in fraction 12 and 13. Trypsin was found to be present as a contaminant in the majority of fractions.

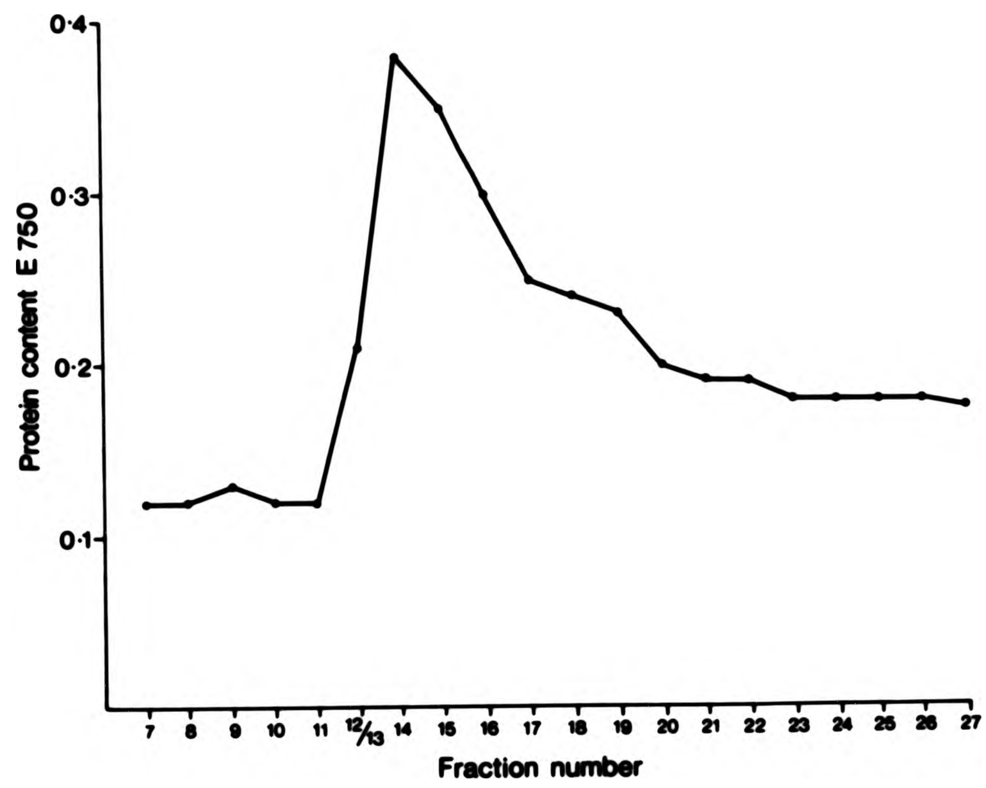
Separation of the T extract on a strongly anionic ion-exchange resin QAE A-50

The crude T extract was eluted with a 2M salt gradient as described. A single chromatographic peak was produced for the absorbance of each fraction at 280nm (Fig. 13). The protein yields for the T-positive fractions were considerably lower (less than 15µg/ml). T-precipitin activity was detected in fractions 14 and 15 as weak lines in gel diffusion. Similarly, as with previous experiments, trypsin was eluted along with the T protein.

3.3.4. PURIFICATION OF THE T PROTEIN BY AFFINITY CHROMATOGRAPHY

The use of affinity chromatography was attempted with a view to separating the T protein from the contaminating enzyme. It has been suggested that the activated groups on the affinity gel (CNBr

Fig. 12 **Ion exchange chromatograms of group G streptococcal T protein on DEAE A-50**



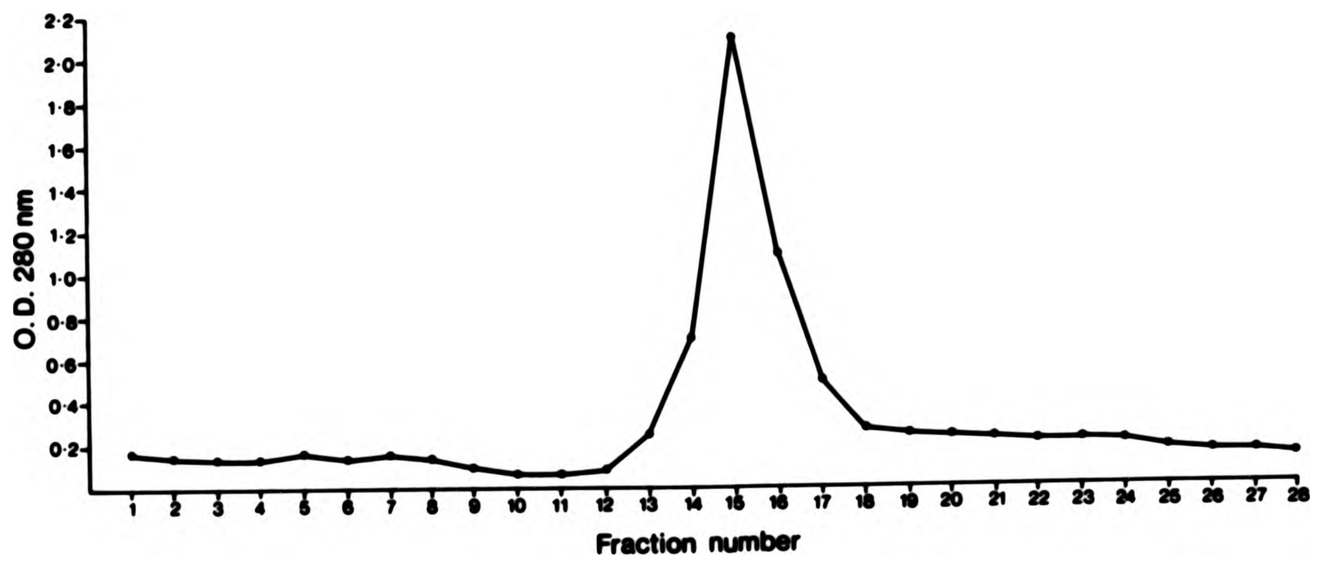


Fig. 13 Ion exchange chromatograms of T protein on QAE A-50

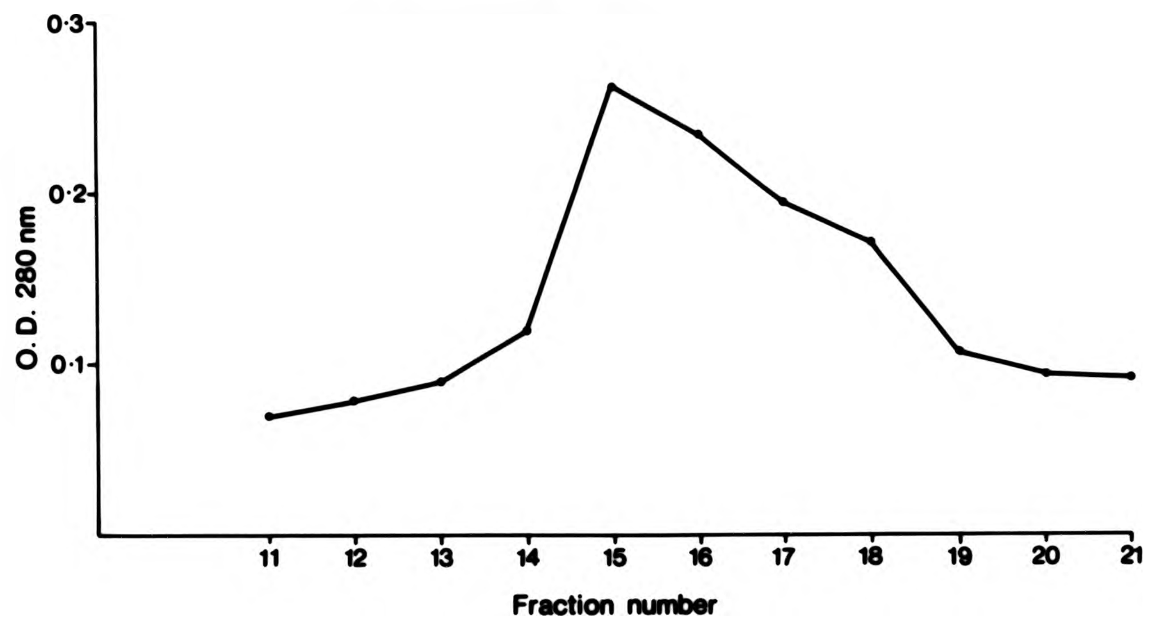
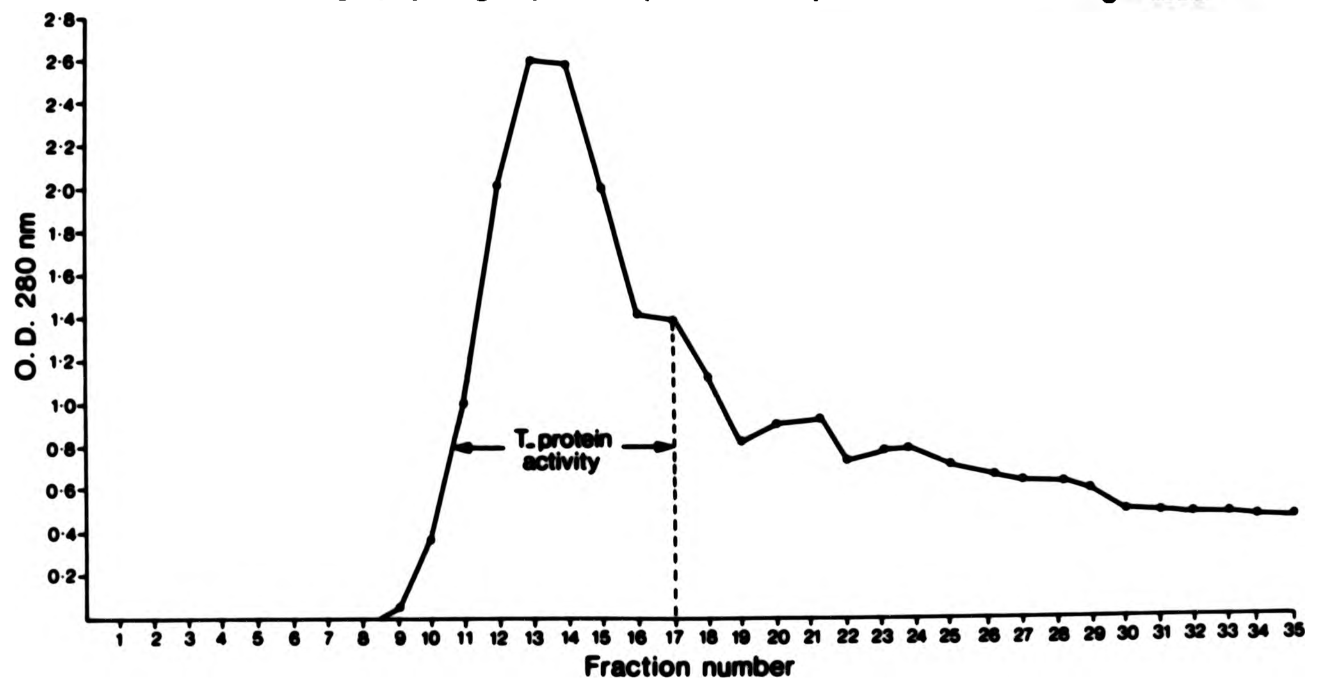
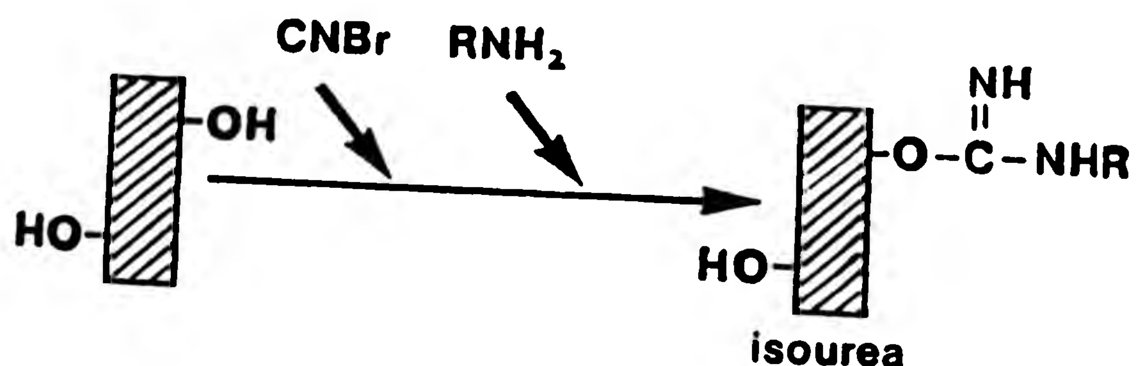


Fig. 14 Chromatography of group G streptococcal T protein on DEAE affi-gel blue



activated Sepharose 4B) react with primary amino groups of the ligand to form isourea (Fig.15). The activation procedure also cross-links the Sepharose and enhances its chemical stability.

Fig.15 Activation and coupling to Sepharose



The ligands used were crystalline trypsin, trypsin inhibitor (Soya bean preparation) and IgG precipitated trypsin antibodies. Thus, three different ligands were coupled separately to preparations of the CNBr Sepharose 4B gel with the aim to achieve a T-protein preparation devoid of trypsin.

<u>Affinity chromatography of the T-protein extract on CNBr activated Sepharose 4B coupled to trypsin antibodies</u>

Antibodies to trypsin could be detected in the majority of antisera that had been prepared using whole cell trypsinised vaccines. In particular, antisera that had been produced against the strain of group G, PT 7118 and group C, PT 3174 were used for the antibody coupling procedures. These sera contained high levels of trypsin antibodies which could be detected as strong precipitin lines in gel diffusion. These antisera did not react with other T-types apart from their own homologous vaccine strain. IgG precipitated

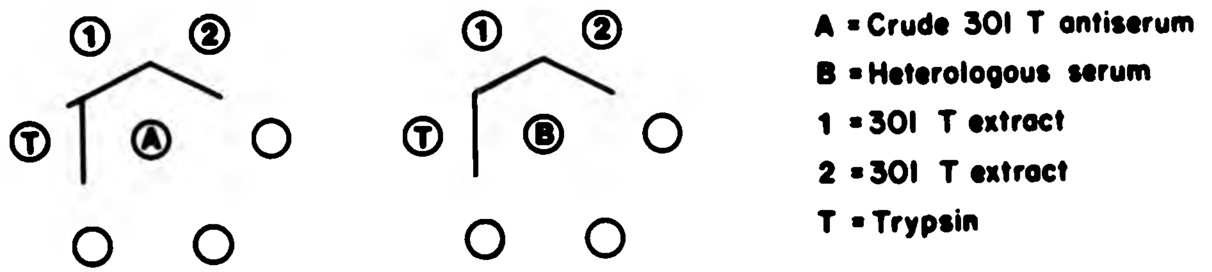
fractions were prepared and coupled to the affinity gels. The degree of coupling was determined by measuring the absorbance of the antibody solution at 280nm before and after coupling. For example, absorbance at 280nm before coupling was 4.03, and after coupling, 2.01 for the Lancefield group G antibody solution. There was slight variation between different coupling procedures. Approximately 50% of the antibody was usually coupled to the gel. The precipitin activity of antibody was also determined before and after coupling (Fig.16).

The void volume of the column was determined with blue dextran (3ml). The fractions obtained were analysed in gel diffusion to determine both T-protein and trypsin activity (Fig.16 and Table 26). Trypsin was detected in fractions 17 - 20 but was not present in the T-protein positive fractions (13-16). This was confirmed by immunodiffusion (Fig.16) and by the negative results on exposed photographic film. The final protein yield for the trypsin-free T-protein preparation was 0.37 mg/ml. Three representative T extracts from each group were purified by affinity chromatography and the separation results achieved were consistent in each case.

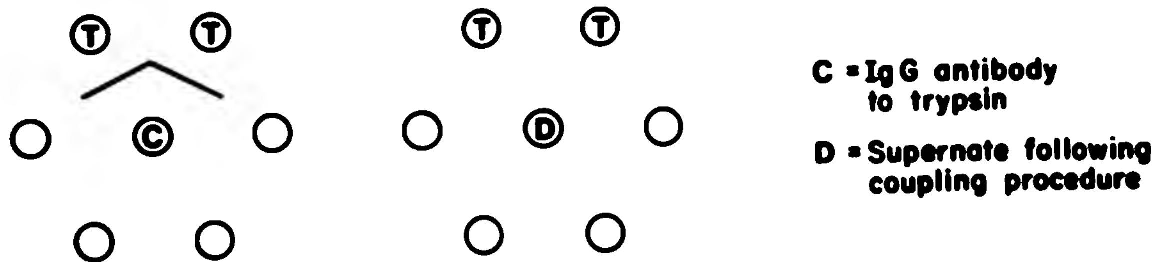
Coupling of trypsin inhibitor to the affinity gel

Trypsin inhibitor was examined as an alternative ligand for the separation of the T protein from the contaminating enzyme (trypsin). As this preparation is a commercially available product (Sigma), it was not necessary to prepare the ligand prior

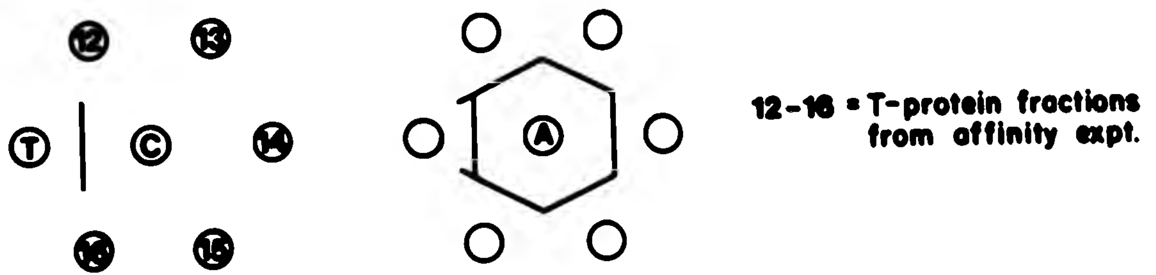
Fig 16. Separation of the T-protein from trypsin by affinity chromatography



I Demonstration by immunodiffusion of trypsin antibodies



II Precipitation activity of the antibody before and after coupling



III T-protein fractions after affinity chromatography on CNBr-Sepharose 4B coupled to trypsin antibodies

Table 26

T protein and trypsin activity in fractions obtained by affinity chromatography of crude streptococcal T protein on CNBr activated Sepharose 4B coupled to trypsin antibodies

Fraction no.	T-protein activity	Trypsin activity
10	-	-
11	-	-
12	-	-
13	+	-
14	+	-
15	+	-
16	+	-
17	-	+
18	-	+
19	-	+
20	-	+
21-40	-	-
41-50	-	-

T-protein and trypsin activity were determined by immunodiffusion.

to coupling. The preparation was coupled directly to the gel. The purpose for the use of trypsin inhibitor was to bind and inhibit any trypsin present in the crude T extract. The crude T extract (0.5ml) was applied to the coupled gel in a column and 0.5 ml fractions were collected and analysed (Table 27). Trypsin could not be detected in any of the fractions. T-protein activity was present in the eluates with a protein yield of 0.4 mg/ml, without the presence of the contaminating enzyme.

Coupling of crystalline trypsin

Crystalline trypsin coupled to CNBr-Sepharose 4B was used in a batch adsorption procedure to extract the T-protein antigen from streptococcal cells. Trypsin so prepared could be regenerated and reused. The purpose of this procedure was to obtain a T antigen preparation free of trypsin directly from whole streptococcal cells in one experimental procedure. The cells were digested with the coupled affinity gel at 37°C for 2, 4, 8 and 18h. The samples were dialysed against distilled water, freeze-dried and subsequently tested for T protein, trypsin and group polysaccharide activity. T protein was liberated after 2h digestion and was still immunologically active after 24h. Group polysaccharide could not be detected, however, trypsin was found to be present as demonstrated by a weak precipitin line in gel. The protein content of the active samples was very low (0.1 mg/ml).

Summary

Table 28 describes the efficiency of affinity chromatography in the

Table 27

T-protein and trypsin activity in fractions from an affinity gel coupled to a trypsin inhibitor

Samples		T-protein activity	Trypsin activity
Fractions	1	-	-
	2	-	-
	3	-	-
	4	-	-
	5	-	-
	6	-	-
	7	+	-
	8	+	-
	9	+	-
	10	+	-
	11	+	-
	12	-	-
	13-20	-	-
Crude T extract		+	+
Trypsin		-	+

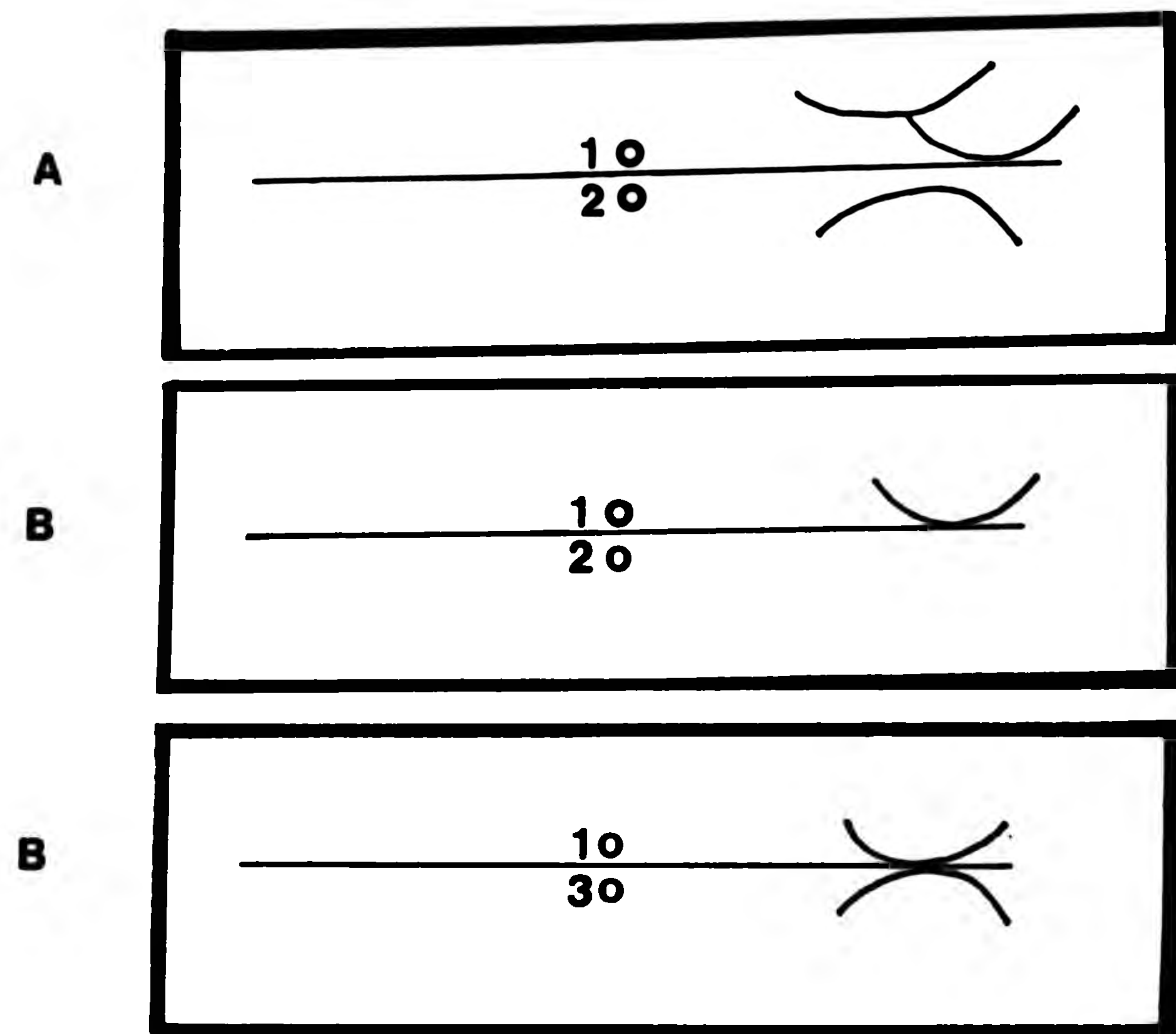
Table 28

Summary of purity and yields of T protein obtained by various methods

Method of preparation	Antigenic activity			Protein content (mg/ml)
	T	Trypsin	Group	
Digestion of cells with crude trypsin	+	+	+	not done
Acid precipitated T extract	+	+	+	2-4
Digestion by affinity gel linked to trypsin	+	+	-	<0.1
*Insoluble trypsin linked to polyacrylamide	+	-	-	0.2
*Affinity gel linked to trypsin antibodies	+	-	-	0.3-0.4
*Affinity gel coupled to trypsin inhibitor	+	-	-	0.4-0.5

*The T-protein preparation was initially digested with trypsin followed by acid precipitation of the protein prior to affinity chromatography.

Fig.17 Immunoelectrophoresis to demonstrate the removal of trypsin from the crude T-protein



A: Homologous antibody

B: Heterologous antibody

1: T-protein extract 308T

2: Trypsin free 308T

3: Trypsin

preparation of a trypsin free T-protein preparation. All methods, with the exception of the direct digestion procedure using crystalline trypsin, produced an enzyme free preparation. However, the protein yields were somewhat low. The affinity gel coupled to trypsin inhibitor produced the highest yield of trypsin free T protein. The purity of the T-protein preparation was further examined by countercurrent immunoelectrophoresis (Fig. 17). Further confirmation on the purity of the preparation was illustrated by polyacrylamide gel electrophoresis on high density gradient gels (see Section 3.3.7.).

3.3.5. SEPARATION OF T PROTEIN ON A COMBINED AFFINITY AND ION EXCHANGE RESIN

DEAE Affi-gel blue (Bio-Rad) is stated to be both an affinity and an ion-exchange gel. It is said to be an improvement over the two step procedure of ammonium sulphate precipitation. The purpose for its use in this study was to assess the function of the gel in the purification of the T protein. By a one step purification procedure a higher protein yield could perhaps be obtained. The other possible advantage in the use of this material was that complex elution schemes were said to be unnecessary because the material is highly selective. Also, the same column could be used repeatedly. The proteins were eluted with 2M salt gradient. Fractions were collected and analysed for T-protein and trypsin activity. T protein and the presence of trypsin was demonstrated. Trypsin was present in fractions 8 to 35, and T-precipitin activity was in fractions 9-17. Protein content was demonstrated as a

single peak by plotting the absorbance for each fraction at 280nm (Fig. 14). The protein yield of T-active material was greater (300 µg/ml) than the yields from the gel filtration and ion exchange separations. But as with these previous experiments trypsin was present in all the T-protein positive fractions.

3.3.6. HYDROXYLAPATITE CHROMATOGRAPHY OF T PROTEIN

As this method of purification had been reported to be successful in the purification of protein antigens for Lancefield group A (Johnson 1975), it was decided to explore the use of this preparation in the purification of Lancefield group C and group G T proteins. Hydroxylapatite column chromatography by stepwise elution of the protein with 0.01, 0.1 and 0.3M sodium phosphate, pH 6.7 was performed. Fractions were collected and analysed as previously. Fractions 1-20 were eluted with the 0.01M buffer, 21-36 with 0.1M and 37-61 with 0.3M. Trypsin and T-protein activity were detected in fractions eluted with the 0.3M buffer solution (fractions 50 and 51). The protein yield was however, very low (25 µg/ml). Hydroxylapatite chromatography has been reported as useful in the purification of group A streptococcal cell wall proteins, notably the M and R proteins, but it did not appear to be of use with group C and group G cell wall T proteins.

3.3.7. DETECTION AND LOCALISATION OF THE T PROTEIN BY POLYACRYLAMIDE GEL ELECTROPHORESIS

The crude T-protein extracts and the positive fractions obtained from the chromatography experiments were analysed further on PAGE

gels to determine their purity and finally the molecular weight of the T-protein molecule.

PAGE on gradient gels in the presence and absence of sodium dodecyl sulphate (SDS)

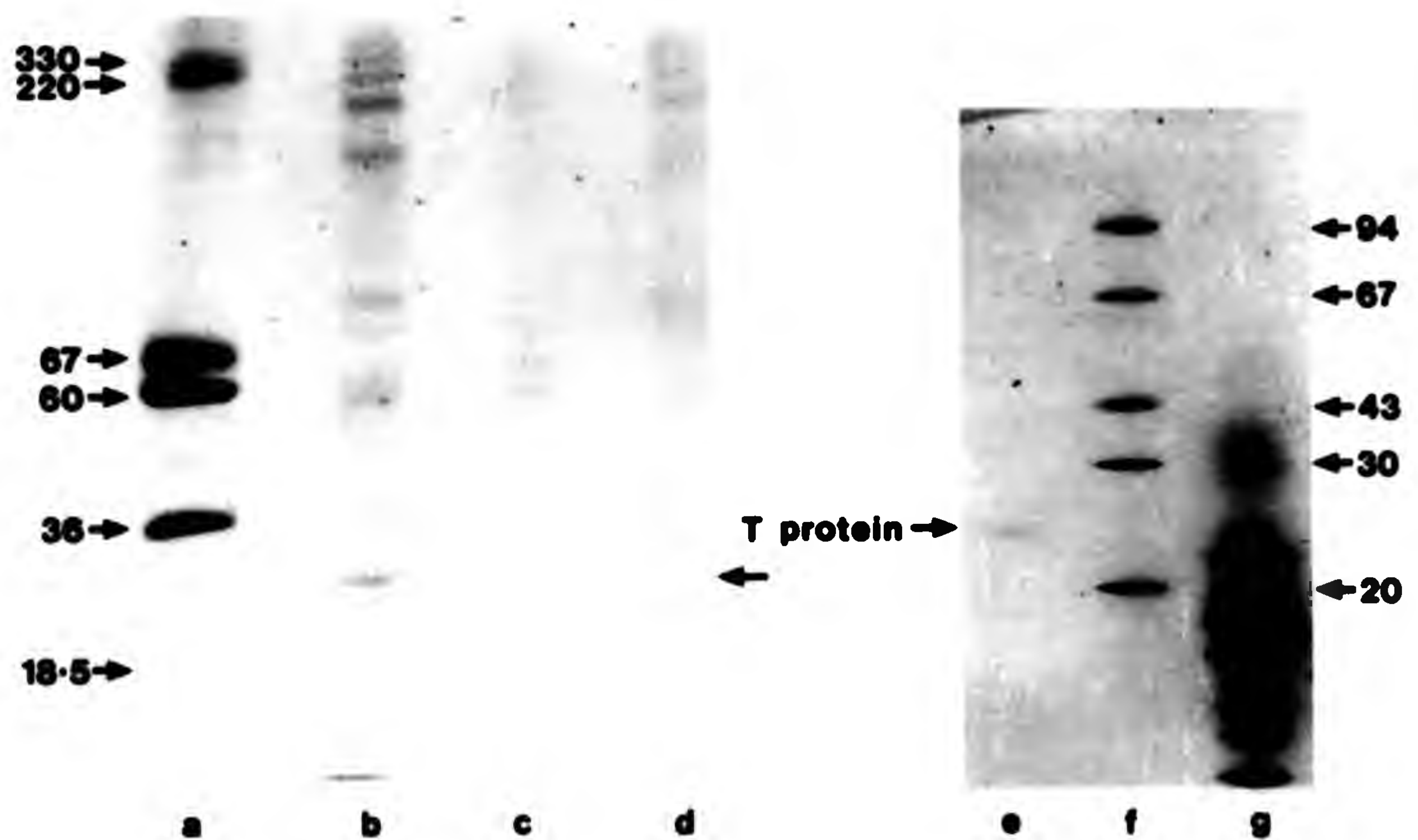
Trial experiments were performed on commercially prepared gradient gels so as to compare the unknown protein extract with proteins of known molecular weights. Experiments were also carried out to determine the various parameters associated with the method, for example, sample volume, protein content, electrophoresis conditions such as voltage, time and buffer pH.

As expected, the crude T-protein preparation contained many impurities especially within the low molecular weight range (14,000-94,400 daltons, range of LMW markers). Therefore the acrylamide gel with a concentration gradient of 2.5-28% (T) was not suitable. So, the higher density gel with a concentration of 2.5-40% (T) was used (this gradient gel concentration was stated to retain molecules up to 10,000 daltons).

Results with the commercially prepared gradient gels were somewhat inconsistent. Many problems were encountered with these high density gradients, the main problem being the polymerisation of the gel during their preparation. Insufficient polymerisation produced air pockets which were only apparent on storage. However, this was the manufacturer's problem and was not encountered with all batches of gels. Using this analytical

method of separation it was possible to monitor the purification procedure of the crude T protein and to obtain an indication of its molecular size. Fig. 18 represents PAGE patterns of crude T protein on high density gradient gels, before purification by column chromatography and eluted protein fractions after chromatography on ion exchange, gel filtration and affinity resins. The chromatography fractions analysed on the gradient gels were active in immunodiffusion against their homologous T-protein antibody. The T-protein positive fractions from the gel filtration and ion exchange columns could not be detected clearly on the gradient gels. This was due to the low protein content of the fractions. Multiple PAGE bands were present in the crude T extract (b). However, samples that had been further concentrated by freeze-drying between each purification procedure could be detected on the gradient gels. Track e on the second gel (Fig. 18) represents the protein profile for the crude T protein after purification by gel filtration and affinity chromatography, and was represented by a single protein band. This fraction was active in gel diffusion against its homologous T antibody. Thus, this protein band is probably the T protein with a molecular weight between 18,500 and 36,000 daltons. The protein profile for trypsin (extraction enzyme) is shown in track g and it is clearly seen that the single T-protein band is absent from this protein pattern. A calibration curve was established with the low molecular weight markers and an estimation of the molecular weight for the presumed T-protein molecule of approximately 26,000 daltons was determined from the graph (see Appendix 2). This was very

Fig.18 Calibration kit proteins and different T protein preparations on high density gradient gels (2.5-40%T)



a: HMW markers x10^3

b: Crude T-protein extract

c: T-protein fraction from DEAE separation

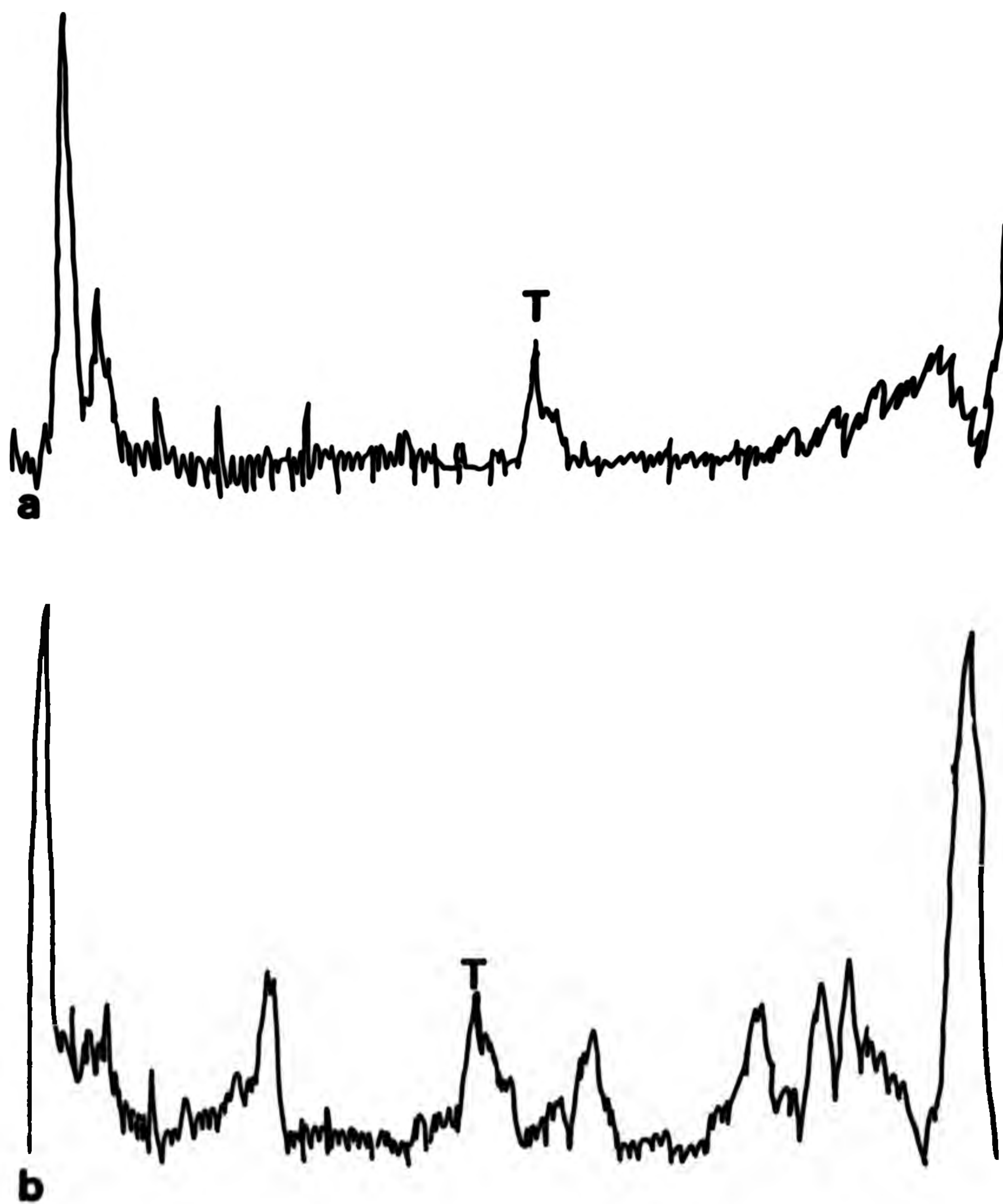
d: Gel filtration G-75 fraction

e: Trypsin-free preparation, following gel filtration and affinity chromatography

f: LMW markers x10^3

g: Crude trypsin

Fig. 19 Densitometric analysis of Coomassie blue stained protein bands of a) Purified T-protein b) Crude T-extract analysed on 2.5-40% gradient gels



closely related to the molecular weight of the extraction enzyme trypsin (23,000 daltons).

The purification procedure for the crude T protein was monitored by densitometry. Fig. 19 illustrates examples of gel tracks analysed by densitometry. The molecular weight markers were clearly indicated (results not shown). The heterogeneity of the crude T protein was also clearly demonstrated by the number of peaks in the densitometer tracing. Peak T was estimated to be the protein peak for the T protein. The purified fraction, which was represented as a faint single band on the gradient gel was illustrated as a small peak in the densitometer scan. The numerous peaks represented by the crude extract were not produced with the purified fraction (Fig. 19).

PAGE of T-protein extracts and whole cell lysates on homogeneous gels in the presence of SDS

Because of the relative inconsistency of the commercial gradient gels due to polymerisation problems by the manufacturers, PAGE on homogeneous gels was used to detect and locate immunologically the T-protein molecule. The molecular weight of the T protein was also determined by this system and the result was compared to that obtained by the previous gel electrophoresis system.

Different concentrations of acrylamide gels were prepared (10%, 12.5%, 15%) and the concentration of 12.5% was suitable for the separation of T-protein extracts for molecular weight

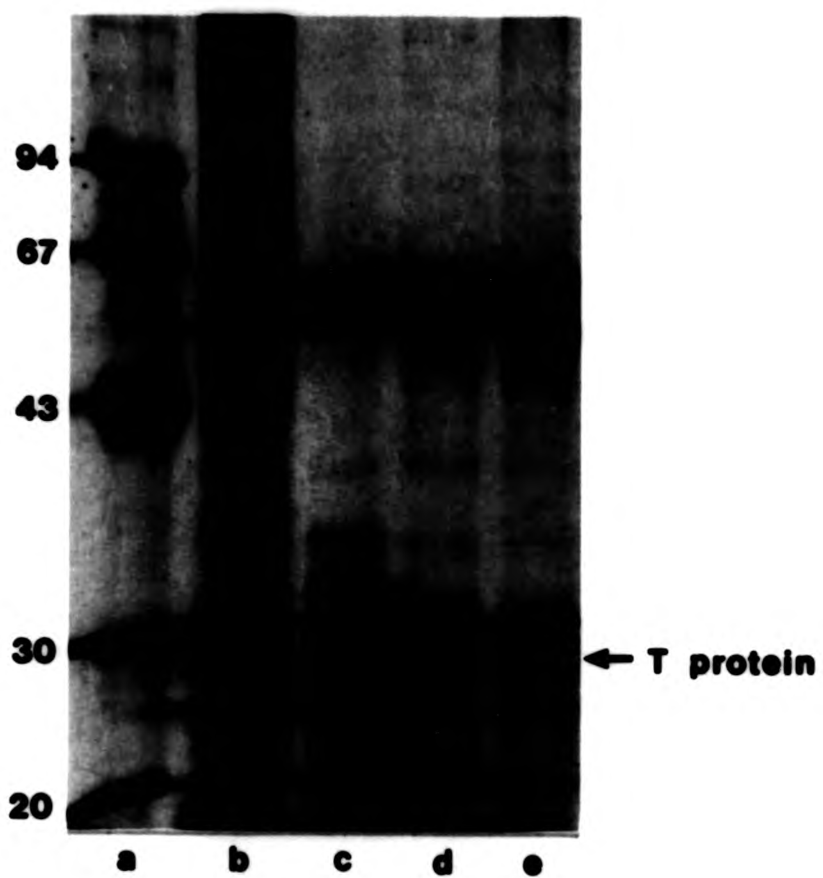
determination. Fig. 20 illustrates the separation of T-protein extracts on 12.5% polyacrylamide gels in the presence of SDS. PAGE patterns of crude T-protein extracts prepared from different serotypes of Lancefield group C and group G streptococci revealed a series of distinct bands within the low molecular weight range of 20,000 to 30,000 daltons. A large proportion of bands were also present in the protein profile for the crude extraction enzyme. This confirmed that contamination of crude T-extracts was largely due to the extraction enzyme. However, one distinct protein band was not present in the protein pattern for the crude enzyme but was present in all T-protein extracts. This band was located in the molecular weight region of 20,000 to 30,000 daltons. As previous PAGE experiments on gradient gels with the purified T protein revealed a low molecular weight product of 26,000 daltons, the distinct band present on the homogeneous gels could be the T protein. This was later confirmed by immunoblotting using an antiserum specific for that particular T protein (see Section 3.3.8.).

Not unexpectedly, whole cell streptococcal lysates of group C and group G strains produced a complexity of protein bands on SDS PAGE. Thus, the need for greater purification in the isolation of the T protein would be necessary.

<u>Electrophoretic mobilities of the standard proteins and the T-protein preparation</u>

The pooled SDS denatured marker proteins were run on each gel.

Fig.20 SDS-PAGE of T proteins on 12.5% slab gels



a: HMW markers x10^3

b: Crude T-protein extract G301

c: Crude trypsin

d: G308T extract

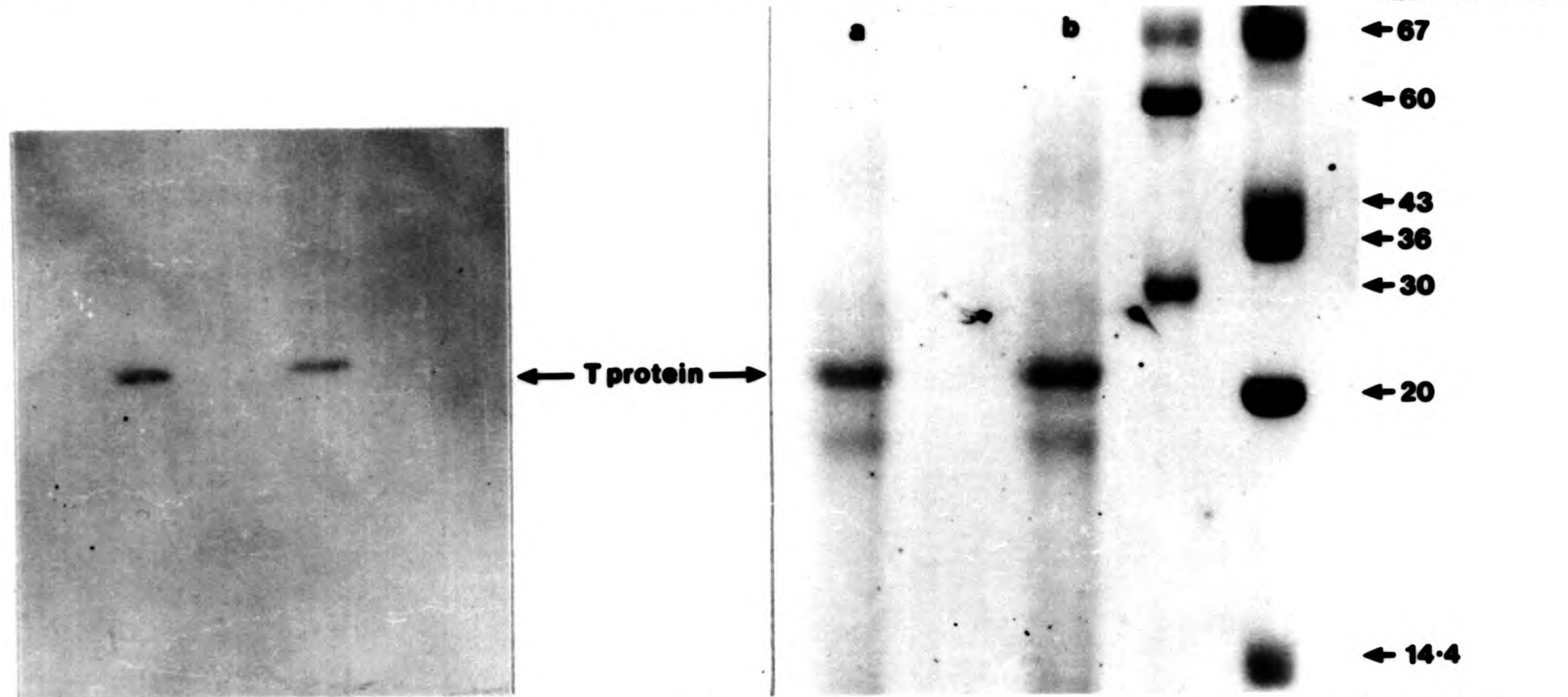
e: C20T extract

Five protein bands developed for the LMW marker and six bands for the HMW marker. The mobilities for each protein were plotted against the logarithm of the molecular weight (see Appendix 2). The linear relationship between the mobility and the logarithm of the molecular weight for each protein was confirmed and the graph was used to estimate the molecular weight of the T protein. In this system, using homogeneous 12.5% polyacrylamide gels the molecular weight was estimated to be 28,000 daltons for the group G streptococcal T protein serotype 301. Brilliant Blue R was the predominant stain used in the PAGE experiments, however, silver staining was also attempted. This did not greatly enhance the resolution of the protein bands.

3.3.8. LOCATION OF THE T PROTEIN BY IMMUNOBLOTTING

T-protein extracts which had previously been separated on 12.5% polyacrylamide gels were transferred by electrophoretic blotting onto nitrocellulose for further immunological studies. The T protein was located immunologically with a polyclonal anti-rabbit T serum specific for that type by immunoblotting with goat anti-rabbit IgG horseradish peroxidase substrate (modified ELISA). T-protein extracts of Lancefield group G streptococcal vaccine strains (serotype 301 and serotype 308) were separated on 12.5% gels and the proteins were subsequently 'blotted' onto nitrocellulose. The stained amido-black nitrocellulose indicated that protein transfer had occurred (Fig. 21). The crude T extracts were revealed as diffuse bands. The location and presence of the T protein as a single band (Fig. 21) for each

Fig. 21 Location of group G streptococcal T protein by immunoblotting



I. Immunoblot detection of T protein

II. Western blot transfer (stained with amido black) of identical T-protein extracts (a and b), LMW and HMW markers

preparation was identified by immunoblotting with specific homologous antisera. These antisera were originally prepared using pepsinised vaccines, thus trypsin antibodies were not present. The molecular weight of the located T protein was within the range of 20,000 to 30,000 daltons and was in agreement with the result previously determined by gradient gel electrophoresis of the purified T protein.

3.3.9. ELECTROMOBILITY OF THE T PROTEIN

Crossed immunoelectrophoresis experiments (at pH 8.2) of partially purified T proteins against their homologous antibody revealed that the mobility of the protein was relatively low. Therefore, these may either be weakly charged or large molecular weight proteins. The question arose whether these proteins might be R proteins rather than T proteins. R proteins are known to be of small molecular weight and may also be antigenic (Johnson 1975). To exclude the possibility of R proteins, crude acid extracts of a selection of strains were tested in double gel diffusion against specifically absorbed T antisera for each particular type. R proteins are not sensitive to hot acid extraction as are the T proteins. Therefore, if the antisera were specific for R proteins, precipitin lines would be produced in gel diffusion. However, if these are true T proteins, a reaction should not occur. Lines of precipitation did not occur with hot acid extracts of these strains, but did, indeed occur with trypsinised extracts of the same strains. Thus these antigens are not R proteins but T proteins.

Estimation of the iso-electric-point of the T protein

The T-protein extracts and trypsin enzyme were focused on a wide pH range, polyacrylamide gel, pH 3.5 to 9.5. After focusing, the pH gradient was determined by means of a surface electrode and the iso-electric point of the T protein was found to be approximately pH 4.5.

Summary

Streptococcal T protein isolated from Lancefield group G streptococci was biochemically purified after initial extraction with trypsin, by gel filtration on Sephadex G-75 and affinity chromatography on CNBr activated Sepharose 4B coupled to trypsin antibodies or to trypsin inhibitor. The purified T protein produced a single precipitin line with its homologous antibody and was devoid of trypsin. The preparation produced a single protein band on high density polyacrylamide gradient gels. The molecular weight of the T protein as determined by two polyacrylamide gel electrophoresis systems and immunoblotting was estimated to be between 26,000 to 28,000 daltons. The protein was therefore found to be a small molecule which was weekly electromobile. The isoelectric point was found to be approximately pH 4.5.

3.4. DETECTION AND ISOLATION OF M PROTEINS IN LANCEFIELD GROUP C AND GROUP G STREPTOCOCCI OF HUMAN ORIGIN

The M protein antigen is believed to be an important virulence factor in Lancefield group A streptococci (S.pyogenes) (Fox 1974). Such strains that possess an M antigen will survive and multiply in normal human blood (Kuttner and Lenert 1944). M-protein antigens have also been identified in some Lancefield group C and group G streptococci. The ability of group C and group G streptococci to survive and replicate in normal human blood and the significance of the presence of both MAP and M antigen in isolates of group C and group G was investigated. The ability of strains to survive and multiply in normal human blood, plus their sensitivity to trypsin is said to verify the true M-nature of the precipitating factor in the antigen extracts of these strains (Lancefield 1962).

3.4.1. SURVIVAL OF GROUP C AND GROUP G STREPTOCOCCI IN NORMAL HUMAN BLOOD

The ability of human isolates of group C and group G streptococci (of known T-protein types) to survive and multiply in normal human blood was investigated by adding a small number of organisms to fresh heparinised blood and assessing their survival.

Initially two different blood donors were used and the results are illustrated in Table 29. The results indicate the failure of some strains to survive and multiply in normal human blood (types 7, 302, 308 and possibly types 21 and 16). This could indicate that

Table 29

Survival of Lancefield group C and group G streptococci in normal human blood

I. Initial Bactericidal Test

Lancefield group	T type	Viable count at 10^{-3}	Survival in blood	
			Donor A	Donor B
C	7	207	7[-]	0[-]
C	20	90	>300[++]	>200[++]
C	21	227	144[+]	0[-]
C	204	133	SC [++++]	SC [++++]
G	16	159	11[-]	60[+]
G	301	>300	SC [++++]	10[-]
G	302	195	39[-]	10[-]
G	308	>300	21[-]	2[-]

II.Bactericidal Test with Survivor Colonies from I (Donor A)

			A	B	C
C	7	400	60[+]	2[-]	9[-]
C	21	250	100[+]	8[-]	0[-]
G	16	300	220[++]	300[++]	50[+]
G	302	180	UC [+++]	UC [+++]	UC [+++]
G	308	300	300[++]	300[++]	35[+]

++++: >500 fold increase in number of survivor colonies
+++ : >100 fold
++ : >10 fold
+ : <10 fold
- : No multiplication

SC: Semi-confluent growth UC: Uncountable colonies

these strains may not possess M-protein antigens or that there were a higher proportion of glossy variants (which are usually the colonial variants devoid of M protein) present. Morphologically, there were, overall, larger numbers of glossy variants in these cultures. Antibodies to M proteins of group C and group G streptococci, could be widely distributed within the population, hence allowing opsonisation and neutralisation of the streptococci by the type specific antibody. This is possibly more evident with the serotype 301 than with the other strains tested. There was greater than a 500 fold increase in the number of surviving colonies of type 301 incubated in donor A but no multiplication at all in the blood of donor B. Hence, donor B may possess type specific bactericidal antibodies to group G serotype 301 (as most of the strains survived and replicated in the blood of donor B). A similar situation was observed with serotypes 7, 21 and donor B. Strains of types 20, 204 and 301 survived in human blood with greater than a 100-fold increase in the numbers of organisms for types 204 and 301.

A second bactericidal test was performed with the survivor colonies of types 7, 21, 16, 302 and 308 with the same two donors plus one additional donor. All the survivor strains subjected to the different samples of blood, survived and multiplied in at least one of the donor blood samples. The survivors from serotypes 16, 302 and 308 survived in all three samples of blood. There was a slight increase in the number of survivor colonies from donor A. These strains did not survive and replicate in the other two

samples. Thus, the bactericidal activity of human blood against the stock streptococcal vaccine strains was somewhat variable. This was enhanced by continually passaging through normal human blood. None of these strains were originally isolated from blood cultures.

It was decided to examine the the survival of group C and group G strains which had been originally isolated from blood cultures of patients with systemic disease. A trial investigation was performed with six strains isolated from blood cultures against four different donor blood samples (Table 30). It appeared that these organisms exhibited a greater degree of virulence with counts increasing by 2 to 200 fold. The blood from donors A and B did not exhibit opsonic properties. However donors C and D exhibited bactericidal activity on strain R85/627 carrying the serotype 20. Donor D also exhibited possible opsonic properties with another additional strain, R85/439. Results obtained with blood culture isolates were more consistent and each strain survived and replicated in the donor blood samples. This led to a further investigation of isolates from a variety of clinical conditions, in order to assess their survival in blood which consequently could be related to the severity of the infection. Overall, 31 different isolates were examined from a variety of sites including those strains described. Table 31 summarises the rate of survival in normal human blood of these isolates from invasive diseases. These infections included, endocarditis, septic arthritis, nephritis, tonsillitis and severe cellulitis. The majority of

Table 30

Survival of streptococci isolated from serious systemic diseases in normal human blood

Strain no.	Lancefield group	T type	Viable count	Survival in blood A	B	C	D
R85/395	G	301	565	>500 ++	>500 ++	>500 ++	80 +
R85/439	G	7/302	500	UC +++	UC +++	500 ++	30 -
R85/873	C	301	280	100 +	UC +++	200 ++	40 +
R85/627	G	20	200	35 +	130 ++	3 -	10 -
R82/2075	G	-	300	UC +++	UC +++	UC +++	UC +++
R82/2520	G	28	300	UC +++	UC +++	300 ++	UC +++

All strains were isolated from blood cultures

Table 31

The survival rate of Lancefield group C an group G streptococci from serious infections in normal human blood

Strain no.	Source	Disease	Lancefield group	T type	Survival in blood (fold increase)
R83/4496	Blood	-	G	16	+++
R85/2038	"	-	"	300	+++
R82/2804	"	Septicaemia	"	301	++++
R85/2801	"	-	"	302	++++
NCTC 11566	"	Endocarditis	"	304	+++
R85/3265	Aspirate	Septic arthritis	"	21	++++
R85/2595	Throat	Tonsillitis	"	16	++++
R85/2631	Skin	Cellulitis	"	303	+++
R85/2144	Skin	Infected burns	"	307	+
R85/2284	"	"	"	307	+
NCTC 11564	"	Cellulitis	C	202	+++
R85/3316	Wound	Septicaemia	"	202	++++
R85/2183	Throat	Tonsillitis	"	204	++
R83/1646	Blood	Endocarditis	"	202	+
R85/573	Blood	-	"	305	++++
R85/873	"	-	"	301	++
R86/882	-	Nephritis	"	204	+++
NCTC 7201	-	Proteinuria	"	308	+++

++++ : > 500 fold increase in number of survivors
+++ : > 100 fold
++ : > 10 fold
+ : < 10 fold

Two samples of blood tested known not to contain M-antibodies to groups C or G

isolates were from blood alone or were isolated from an additional site. The results in Table 32 demonstrate that isolates from blood culture not unexpectedly survived and multiplied in normal human blood. All blood culture strains appeared to possess an M protein-like antigen which allowed resistance to phagocytosis and multiplication in normal blood, presumably enhancing the degree of virulence and invasiveness of the organism in man.

3.4.2. DETECTION OF M-ASSOCIATED PROTEIN (MAP)

MAP is stated to be a 'non type-specific' cellular protein which is closely associated with the M antigen. In the Lancefield group A streptococci it has been identified as a non-specific component of the M-protein molecule itself and is said to be found in all M-positive strains (Widdowson et al. 1971), but is absent from M-negative variants. It has also been identified in group C and group G streptococci but MAP has not been physicochemically defined in these organisms. The presence of MAP in groups C and G streptococci could provide an indication of whether or not an M antigen may be present.

Crude acid extracts prepared from nineteen different strains of group C and group G were examined. The sera from three individuals who did not have a history of recent streptococcal infection were screened against all the extracts in a complement fixation test (CFT). All three sera were previously used in the survival experiments and did not exhibit bactericidal properties with the majority of the strains.

Table 32

The survival of group C and group G streptococci in normal human blood

Strain no.	Lancefield group	Source	T type	Viable count	Survival in blood		
					Donor B	Donor C	Donor D
R83/1646	C	Blood	202	193	103 +	78 +	60 +
R85/3316	C	*Wound	202	400	C +++++	SC ++++	SC ++++
R85/3315	C	*Blood	202	380	C +++++	SC ++++	SC ++++
R85/2038	G	Blood	300	300	UC +++	UC +++	90 +
NCTC 11566	G	Blood	304	300	UC +++	UC +++	SC ++++
R83/4496	G	Blood	16	200	UC +++	SC ++++	UC +++
R82/2804	G	Blood	301	300	C +++++	C +++++	C +++++
Control	A	stock strain	MT22	300	SC ++++	SC ++++	SC ++++

* : Isolates from the same patient

+++++ : >1000 fold increase = confluent growth
++++ : >500 " " " semi-confluent growth
+++ : >100 " " " uncountable colonies
++ : >10 " "
\+ : <10 " "

The serum from donor I at a dilution of 1 in 20 fixed complement with 15 of the extracts at dilutions ranging from 1 in 10 to 1 in 80. Fixation titres of more than 1 in 40 were observed with only four of the extracts. Serum II fixed complement with 13 of the antigens with three producing titres of greater than 1 in 40. The serum from the third donor was not active at all (Table 33). Additionally, Table 33 illustrates the differences in MAP titres between the different antigenic extracts. For example G301BC produced titres of 1 in 80 with the sera from Donors I and II, whilst G307 produced a titre of 320 with donor II serum and only 40 with donor I.

Detection of MAP in sera from patients with recent streptococcal infections

The presence of antibodies to MAP in patients sera, which may be indicative of past infection by either group A, group C or group G streptococci possessing M-protein antigens, was investigated. The nineteen extracts examined in the previous section were tested for the presence of complement fixing properties against samples of sera from four patients with severe infections.

Necrotising fasciitis was diagnosed in patient I and a group G streptococcus serotype 7/302 was isolated from the skin. Three serum samples were taken during the course of this patient's illness, serum A at onset, serum B eight days later and serum C, seventeen days later. Patient 2 presented with a purpuric and

Table 33

The difference between strains of group C and group G streptococcal acid extracts in complement fixation tests with the sera of three human donors

Strain	Lancefield group	C F T (M A P) titre in donor sera I	II	III	negative control
*301BC	G	80	80	0	0
7/302BC	"	10	20	0	0
20BC	"	20	10	10	0
T-veBC	"	80	40	0	0
28BC	"	40	0	0	0
301	"	80	40	0	0
302	"	40	10	0	0
303	"	10	10	0	0
304	"	20	40	0	0
305	"	0	0	0	0
306	"	20	20	0	0
307	"	40	320	0	0
308	"	20	160	0	0
7	C	0	0	0	0
20	"	80	0	0	0
21	"	0	0	0	0
200	"	0	0	0	0
201	"	40	20	0	0
301BC	"	10	20	0	0

*BC: blood culture isolates
Types 301 - 308 and 7 - 201 were stock strains with known T types.

vesicular rash from which no organisms were isolated. Serum samples were taken twenty one days apart, but not at the onset of illness. Patient 3 had severe cellulitis of the leg and a single serum sample was taken during the illness. Patient 4 presented with endocarditis and one sample was submitted for examination. These patients were not related in any way.

Complement fixing antibodies (possibly MAP), were present in the serum samples from patients 1, 2 and 3, with the exception of the first sample from patient 1. The second sample produced significant titres with nine of the antigens and the complement fixation antibodies fell significantly in the third sample. Elevated titres were not as apparent with the other samples (Table 34). Antibody to MAP was not present in the serum sample from the fourth patient (serum G).

Relationship of antibody to MAP with other streptococcal antibodies

The relationship of the MAP antibody titre in patients with streptococcal infection to antibodies against other streptococcal extracellular substances was clearly demonstrated (Table 35). The antibody levels to cellular products such as MAP take longer to emerge and remain raised for longer periods, than antibodies to extracellular products, such as streptolysin O (ASO), DNase B (ADB) and hyaluronidase (AHT) (Widdowson and Wormald 1981). Serological tests against these extracellular products were performed on the seven serum samples previously discussed and the results were compared to the MAP titres obtained. These additional serological

Table 34

Detection of MAP antibodies in patients with streptococcal infections

Antigen extracts	Patients sera (CFT titre)						
	A	B	C	D	E	F	G
G 301BC	0	80	40	40	40	40	40
G 7/302BC	0	80	40	10	10	40	20
C 301BC	0	40	20	10	20	10	10
G 20BC	0	40	20	10	20	10	10
G T-veBC	0	40	40	20	20	20	20
G 28BC	0	160	80	40	40	80	40
G 301	0	>320	80	80	80	80	40
G 302	0	40	20	10	10	10	20
G 303	0	40	40	40	40	20	20
G 304	0	80	40	20	20	40	20
G 305	0	40	20	0	0	20	20
G 306	0	80	40	20	40	20	20
G 307	0	80	40	20	40	20	20
G 308	0	40	20	20	20	20	20
C 7	0	10	10	0	0	10	10
C 20	0	160	160	40	80	80	40
C 21	0	20	20	0	0	0	10
C 200	0	20	10	0	0	10	10
C 201	0	80	20	20	20	20	20

Table 35

The significance of MAP to other antibody tests as indicative of group C or group G streptococcal infection

	Serum sample	Antibody titres				
		ASO	ADB	AHT	AHT C/G	MAP
Patient 1	A	140	50	32	< 10	-
	B	>800	75	32	20	+
	C	>800	100	32	40	+
Patient 2	D	>800	<50	32	320	+
	E	>800	<50	<32	160	+
Patient 3	F	720	75-100	128	320	+
Patient 4	G	100	<50	32	<10	-

Significant Titres

(200)	ASO	=	Anti-streptolysin O
(250)	ADB	=	Anti-deoxyribonuclease B
(128)	AHT	=	Anti-hyaluronidase test specific for group A
(10)	AHT C/G	=	Anti-hyaluronidase test specific for group C and group G streptococci

tests were carried out by Miss G.Hallas of the Streptococcus Reference Unit, Central Public Health Laboratory, London. The results indicated that the serum samples were from patients with group C or group G streptococcal infection (Table 35). In all cases the titre to streptolysin O was raised. Streptolysin O is identical for groups A, C and G streptococci (Alouf 1980). The antibody titres to ADB and AHT were not significant, whereas antibodies to the AHT specific for group C and G streptococci were raised except in the early onset serum of patient one. Patient 4 produced very low antibody titres. This patient had endocarditis. It was unusual to find such low titres as patients with endocarditis produce very high titres of both complement fixing and opsonic antibodies for the causative organism present (Laxdal _et al._ 1968). It may be that the serum sample from this patient was an early onset sample or in fact taken whilst the patient was undergoing antibiotic therapy. Overall, the presence of antibody to MAP is stated to correlate well with the other serological tests.

Relationship between presence of MAP and the survival of streptococci in normal human blood

The results indicated the inconsistent relationship between MAP and the survival of these streptococci in normal human blood. The hypothesis that high MAP titres are a good indication of the presence of M protein-like antigens could not be substantiated with group C and group G streptococci (Table 36). The survival and replication of these strains in normal human blood appeared to be a

Table 36

Relationship of the M-associated protein (MAP) titre to the survival in blood

Lancefield group	T type	MAP titre	Survival in blood
* G	301	80	++
* G	7/302	20	+++
* G	20	20	++
* G	-	80	+
* G	28	40	+++
* C	301	20	+++
G	301	80	+++
G	302	40	-
G	303	10	±
G	304	40	+++
G	305	0	-
G	306	20	+
G	307	160	+
G	308	160	++
C	7	0	-
C	20	80	++
C	21	0	+
C	200	80	+
C	201	80	+

* These strains were isolated from blood cultures.

more useful indication of virulence. If however the proposition that possession of MAP is indicative of M protein is correct, then those strains with low MAP titres which survive in blood may possess another antiphagocytic factor. It has been suggested (Becker et al. 1973) that group A streptococci possess factors other than protein or hyaluronic acid which behave as determinants of virulence and this might also be true for groups C and G streptococci.

3.4.3. ISOLATION OF M-PROTEIN ANTIGENS AND THE PRODUCTION OF OPSONIC ANTISERA

Strains showing positive results in the bactericidal tests and MAP complement fixation tests were selected for the isolation of possible M protein-like antigens to be used for the production of opsonic antisera to these streptococci. Candidate strains for vaccine preparation were predominantly isolates from blood cultures and systemic disease (Table 5). Whole cell saline suspensions of streptococci are used as the conventional vaccines in the preparation of M-typing sera for Lancefield group A. As a first step, it was decided to explore the use of such vaccines in the preparation of M-typing sera for group C and group G streptococci. Twenty one vaccine strains were used, 13 belonging to Lancefield group G and 8 belonging to Lancefield group C. Over an eight-week period of immunisation, antibodies were stimulated to both the group polysaccharide,and the T and M proteins. The degree of the response against each antigen varied between strains. These findings are illustrated in Table 37. The main disadvantage in

Table 37

Antibody response to the M protein using whole cell vaccine preparations

Strain	Lancefield group	Type	Antibody response		
			Group	T protein	M protein
NCTC 11564	C	202	++ (3)	+++ (3)	+ (4)
NCTC 11629	C	204	+++ (4)	++ (4)	+ (4)
R86/882	C	204	+ (3)	+ (3)	+ (3)
R85/573	C	305	+ (4)	+ (4)	+ (4)
R86/273	C	308	+++ (3)	+++ (3)	+ (3)
NCTC 4540	C	7	+ (3)	+++ (3)	+ (6)
NCTC 5370	C	20	++ (4)	+++ (4)	+ (5)
NCTC 5371	C	21	+ (6)	+++ (3)	+ (7)
NCTC 5969	G	16	++ (4)	+ (4)	-
R85/2595	G	16	+++ (3)	++++(3)	+ (5)
R83/4496	G	16	+++ (3)	++++(3)	+ (5)
R85/627	G	20	+++ (5)	+++ (5)	+ (5)
R85/3265	G	21	+ (5)	+++ (5)	+ (5)
R85/2038	G	300	+++ (3)	+++ (3)	+ (3)
R82/2804	G	301	+ (5)	+++ (5)	+ (5)
NCTC 11555	G	301	+++ (6)	++ (6)	-
R85/439	G	7/302	+ (3)	+ (3)	+ (3)
R85/2631	G	303	+ (3)	+ (3)	+ (3)
R85/3144	G	307	++++(6)	++++(6)	+ (6)
R85/2284	G	307	++ (4)	++ (5)	-
NCTC 11630	G	308	++ (5)	+++ (5)	+ (5)

( ): week during inoculation programme when peak antibody response is obtained.
++++: antibody titre >1/500
+++ : " " >1/100
++ : " " >1/10
+ : " " <1/10

Group and T-antibody titres = agglutinin titres .
M-antibody titre = precipitin titres.

the use of whole cells was the absorption procedure required for each serum. The group polysaccharide antibody often masked any type-specific antibody present. Antibodies to M protein-like antigens could be detected by double gel diffusion using crude acid extracts of homologous and heterologous antigens (see Section 2.8.4.). Precipitin reactions could be detected with the sera taken between weeks 3 and 7 of the inoculation programme for most strains. The gel precipitin titres were usually within the range of 1 in 2 to 1 in 4. From these results it would seem that strains which survived and multiplied in normal human blood did produce an immunogenic response in rabbits to an M protein-like antigen. Therefore, survival in blood was accepted as the indicator of the presence of such proteins in an immunising strain (Table 38).

The isolation of the M-protein antigen

Ammonium sulphate fractionation of crude acid extracts of streptococci prepared from 2l of broth culture (per strain) was attempted as a means to separate and isolate the M protein from the group polysaccharide antigen. The overnight growth of 2l of Todd Hewitt broth yielded approximately 2g of whole wet streptococci. Crude acid extracts produced approximately 30 to 36mg of protein from the 2g for a yield of 15 to 18mg of protein per g wet weight of streptococci. Fractional precipitation was carried out using ammonium sulphate. Heavy precipitation occurred at each step especially at concentrations greater than 40% saturation. The fractions were analysed for the presence of group and M antigens.

Table 38

The survival of Lancefield group C and group G streptococci in normal human blood and the presence of M protein

Strain no.		Source	Lanc. group	T type	Survival in blood	Immunogenicity in rabbits
NCTC 11564		Skin	C	202	+++	+
NCTC 11629		Throat	C	204	++++	±
R86/882		Nephritis	C	204	++++	+
R85/573		Blood	C	305	++++	+
R86/273		Blood	C	308	+++	+
NCTC 4540		Throat	C	7	+	+
NCTC 5370		Throat	C	20	++	+
NCTC 5371		Throat	C	21	++	-
NCTC 5969	*	Throat	G	16	-	-
R85/2595	†	Throat	G	16	++++	+
R83/4496	‡	Blood	G	16	++++	+
R85/627		Blood	G	20	+	+
R85/3265		Arthritis	G	21	++++	±
R85/2038		Blood	G	300	+++	+
R82/2804		Blood	G	301	++++	+
NCTC 11555		Eye	G	301	++++	-
R85/439		Blood	G	7/302	+++	+
R85/2631		Cellulitis	G	303	+++	+
R85/2144		Raw area	G	307	+	±
R85/2284		Raw area	G	307	+	-
NCTC 11630		Perineum	G	308	-	+

* Normal throat carriage
† Pharyngitis
‡ Septicaemia

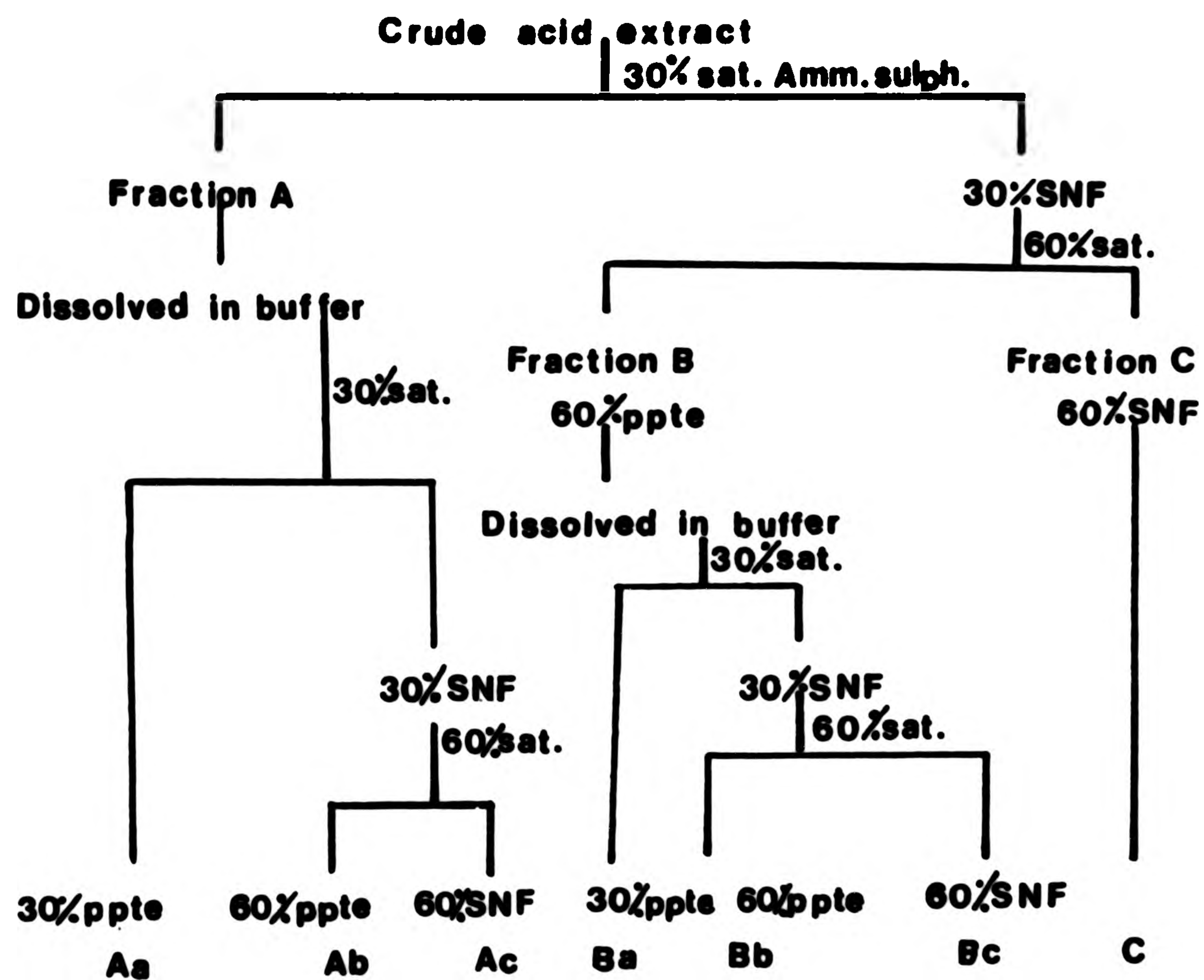
++++ 500 fold increase in the number of survivor colonies
+++ 100 " "
++ 10 " "
+ 10 " "
- No multiplication

The group polysaccharide was detected in all fractions. Separation of the M protein and group polysaccharide was then attempted by fractional precipitation with ethanol but again the two could not be separated. Previous workers (Johnson and Vosti 1968) described a procedure for the purification of Lancefield group A streptococcal M protein which involved precipitation with $(NH_4)_2SO_4$ at 33 to 60% saturation. Proteins that precipitated were dialysed and the M protein was separated from the group antigen, and further purified, by column chromatography. As antisera to the M proteins were then not available for the group C and group G streptococci, a group G streptococcal strain carrying the group A type 12M antigen was used as a 'marker' for comparison against the other two strains examined (group G, 308 and group C type 21). The fractionation procedure used is illustrated in Table 39. The results (Table 40) indicate that group antigen was present in all fractions with the exception of the precipitates obtained at 30% and 60% saturation stages (Ab and Ba). Therefore, it appeared that the M protein may have been precipitated at 60% saturation with $(NH_4)_2SO_4$. This was very similar to those results obtained with Lancefield group A streptococcal extracts (Johnson and Vosti 1968). The fractions containing M antigens were pooled for each strain and the protein content found to be low (from 50 to 75 µg). Rabbits were inoculated with these fractions but produced no detectable antibody. Their immunogenic potential was apparently poor.

In an attempt to prepare an immunogenic protein extract devoid of

Table 39

The isolation of group C and group G streptococcal M proteins by ammonium sulphate fractionation of crude acid extracts



% sat. Amm.sulph. : % saturation with ammonium sulphate

SNF : Supernatant fluid

ppte : Precipitate

Table 40

Antigenic content of ammonium sulphate fractions of group C and group G streptococci

Fractions	Strains			
	Type 12		Type 308*	Type 21*
	group	M	group	group
Aa	-	-	-	-
Ab	-	+	-	-
Ac	+	-	+	+
Ba	-	+	-	-
Bb	+	+	+	+
Bc	+	-	+	+
C	+	-	+	+

* M sera not available at this stage for these types.

group polysaccharide yet containing M protein, crude extracts for six strains of streptococci were concentrated and subjected to affinity chromatography. An affinity chromatography column with group polysaccharide antibodies coupled to CNBr-Sepharose 4B was prepared. The crude acid extracts were separated on the affinity gel and the fractions were analysed for the presence of type-specific M protein using, if available, specific antisera. The results are shown in Table 41. The fractions obtained in the void volume appeared to contain the group polysaccharide antigen and did not bind to the affinity gel. This could be an insoluble portion of the group polysaccharide present. Rabbits were inoculated with pooled and concentrated fractions as follows:-

Rabbit number	Strain	Fraction used for vaccination	Expected content
1	C21	8 - 14	M protein
2	G308	1 - 5	Group antigen
3	G308	15 - 18	Group antigen
4	G12	9 - 14	M protein
5	G302	8 - 15	M protein
6	G301	9 - 15	M protein
7	C20	10 - 15	M protein
8	G308	8 - 13	M protein

Rabbits 2 and 3 were inoculated with fractions of G308 eluted at different stages of the chromatography experiment and both pooled fractions were found to contain the group polysaccharide. The other rabbits were inoculated with purified extracts which could have contained M protein. M-protein activity was demonstrated in

Table 41

Detection of group and M antigens from crude acid extracts of group C and group G streptococci after affinity chromatography on CNBr Sepharose 4B coupled to a heterologous antiserum devoid of M-protein antibody

Crude acid extracts	Presence of group antigen																	
	Fractions																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
G 12	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
*M protein	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
G 308	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+
*M protein	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
C 21	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+
C20	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
G 302	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

* M sera available for these two serotypes.

gel diffusion for types 12 and 308. Rabbits were inoculated subcutaneously with purified antigen plus aluminium hydroxide adjuvant in two sites. After two weeks, intravenous injections were given weekly for four consecutive weeks.

Consistent results were obtained for each animal (Rabbits 1-8). None of the purified fractions induced an antibody response. These fractions could have been soluble antigens which were quickly excreted by the animal. These results support those of Barkulis and Jones (1957) who stated that acid extracts of the group A M protein are very weakly or not at all immunogenic. Thus M-protein antigens can be released by acid extraction of streptococci but they did not have the immunogenic potential to induce antibody production in animals, under the conditions of immunisation applied here.

Peptic digestion of streptococcal M proteins

Early reports have indicated that the type-specific M-protein antigen of group A streptococci is extremely sensitive to tryptic or peptic digestion (Lancefield 1928, 1943). Other workers reported the use of a mild peptic digestion procedure for their extraction (Beachey et al. 1974). The M proteins released were said to be highly immunogenic and devoid of group polysaccharide antigen. This method was therefore employed for the attempted production of M-protein antigens from group C and group G streptococci.

Determination of the optimal conditions for the release of M protein from streptococci by peptic digestion

For the group A streptococci, optimal amounts of type-specific M protein were stated to be released after 20min of digestion with 0.02mg of pepsin per ml at pH 5.8 (Beachey et al. 1974). For the determination of optimal pH, pepsin concentration and enzymatic digestion time required for the extraction of M protein, a Lancefield group G strain, serotype 308 was used (Table 42). M protein was detected by double gel diffusion with absorbed homologous antisera. Each extract was tested in addition for the presence of T protein and group polysaccharide. The results indicated that group and T protein could not be detected in the extracts. Precipitin activity was however present in all digests released within the pH range of 4.5 to 7.0 at both concentrations of pepsin. When narrower pH intervals between pH 5.0 and 6.6 were employed, the highest titre of M antigen (1 in 8) was obtained at pH 5.8 after 30 min digestion at 37°C. The effect of pepsin concentration at pH 5.8 on the amounts of M protein extracted at 30 min was determined by immunodiffusion of the extracts against homologous M-type specific antiserum. The largest precipitin ring was obtained at a concentration of 50 µg of pepsin per ml. It was noteworthy that two patterns of digestion occurred:-

a) One producing sharp precipitin lines of complete identity with their homologous crude acid extract, with a faint inner line forming a line of partial identity with the control acid extract. An inner line was not produced by the acid extracted streptococci.

Table 42

Experiment to determine the optimum conditions for the peptic extraction of group G streptococcal M protein serotype 308

I. Pepsin concentration 20μg/ml

pH	Time (minutes)					
	10	20	30	60	120	180
2.0	-	-	-	-	-	-
3.0	-	-	-	-	-	-
3.5	-	-	-	-	-	-
4.0	-	-	-	-	-	-
4.5	(+)	(+)	(+)	(+)	(+)	(+)
5.0	(+)	(+)	(+)	(+)	(+)	(+)
5.2	+	+	+	(+)	(+)	(+)
5.6	+	+	+	(+)	(+)	(+)
5.8	+	+	+	+	(+)	(+)
6.0	+	+	+	+	+	+
6.6	(+)	+	+	+	+	+
7.0	(+)	(+)	(+)	(+)	(+)	(+)

II. Pepsin concentration 50μg/ml

pH	Time (minutes)					
	10	20	30	60	120	180
2.0	-	-	-	-	-	-
3.0	-	-	-	-	-	-
3.5	-	-	-	-	-	-
4.0	-	-	-	-	-	-
4.5	(+)	(+)	(+)	(+)	(+)	(+)
5.0	(+)	(+)	(+)	(+)	(+)	(+)
5.2	+	(+)	(+)	(+)	(+)	(+)
5.6	+	+	(+)	(+)	(+)	(+)
5.8	+	+	+	+	(+)	(+)
6.0	+	+	+	+	(+)	(+)
6.6	+	+	+	+	+	+
7.0	(+)	(+)	(+)	(+)	(+)	(+)

(+): Non-specific M protein
 + : M protein

b) Pepsin extracts that produced a single faint line of partial identity with the control acid extract. This may indicate the release of a non-specific protein moiety (Table 42), possibly related to MAP.

The effect of digestion time upon the release of protein material is shown in Table 42. It would appear that the release of M protein was determined by pH and digestion time.

To confirm that pepsin was the enzyme responsible for the digestion process and the release of M protein from the cell wall, digestion for 30 min was repeated at pH 5.8 and 37°C with and without the presence of pepsin (50 µg). The digests obtained were tested in gel diffusion for the presence of group, M and T antigens. The pepsin digest produced double precipitin lines in gel diffusion, a sharp outer precipitin line with an additional very weak inner line. The cells incubated in buffer alone did not yield precipitates in gel diffusion tests. T protein and group antigen could not be detected (Fig. 22).

Pepsin digests were compared with acid extracts of the homologous type by immunoprecipitation against specific M antisera. A pepsin digest of a strain of type G308 produced double precipitin lines, the inner faint line reacting with the acid extract as a line of partial identity. None of the pepsin digests reacted with heterologous unabsorbed antisera nor with the Lancefield group C or group G specific antisera. In contrast, hot acid extracts produced strong precipitin lines with many heterologous unabsorbed

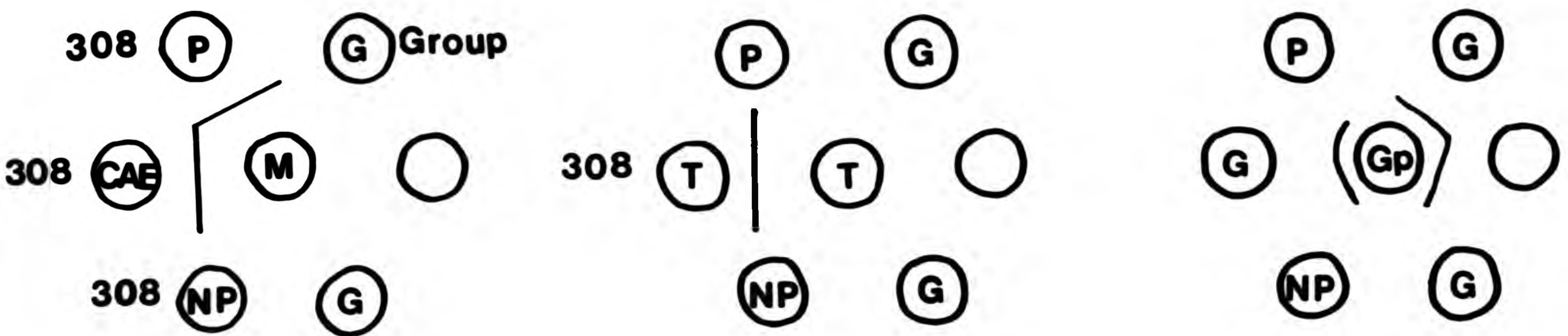


Fig.22 Agar gel diffusion tests of crude pepsin extracts:P, acid extracts:CAE and untreated cells:NP, of serotype 308 against specific M, T and group G antisera

M antisera produced with whole cell vaccines as well as with their group specific antiserum.

Peptic digestion of different serotypes of Lancefield group C and group G streptococci

Peptic digests were prepared from strains of Lancefield groups C and G streptococci and from two control strains with known M type antigens belonging to Lancefield group A. The streptococcal cells were extracted twice with pepsin and their protein contents and precipitin activities compared. A greater yield of protein material was obtained by extracting the cells twice with the enzyme (Table 43). The protein yield obtained varied from 5mg to 13mg per g of wet cells extracted. As in previous experiments, group and T antigens could not be detected. Precipitin lines were however detected in gel diffusion tests against homologous M antisera. In total, 15 pepsin extracts were prepared against 15 strains of group C and group G streptococci.

Immunogenicity of pepsin extracts

Rabbits were immunised with a primary dose of pepsinised extract containing aluminium hydroxide adjuvant, subcutaneously in two sites, followed after two weeks, by single doses of vaccine intravenously for four consecutive weeks. The protein content per dose of vaccine was 400 μg/ml. The total dose of protein injected per rabbit was 2 mg.

Antibodies to the group polysaccharide and M protein were not

Table 43

Comparison of protein yields from first and second extractions of streptococcal whole cells with pepsin

Strain	Weight of wet cells (g)	Protein yield (from 41 culture)		Protein extracted per g of wet cells (mg)	
		1st*	2nd*	1st*	2nd*
G 12	5.4	18.8	10.8	3.5	2.0
G 308	4.4	23.2	>32.0	5.3	>7.0
A 6	5.4	12.8	22.4	2.4	4.1
A 49	5.2	17.2	16.8	3.3	3.2

1st*}
First and second extractions
2nd*}

induced in the rabbits. Even though M-preciptin activity was present in the pepsin extracts the use of these extracts in an aluminium hydroxide adjuvant did not produce an immune response to the M protein. However, the pepsin preparations did stimulate the production of antibodies to the T protein, although this protein was not detectable by slide agglutination or precipitation in the initial pepsin extract. Homologous trypsinised whole cells of streptococci produced agglutinin titres with the T-protein antisera ranging from 1 to 16 (Figs. 8 and 9). Thus, pepsin extracts in an aluminium hydroxide adjuvant did not stimulate antibodies to the M protein.

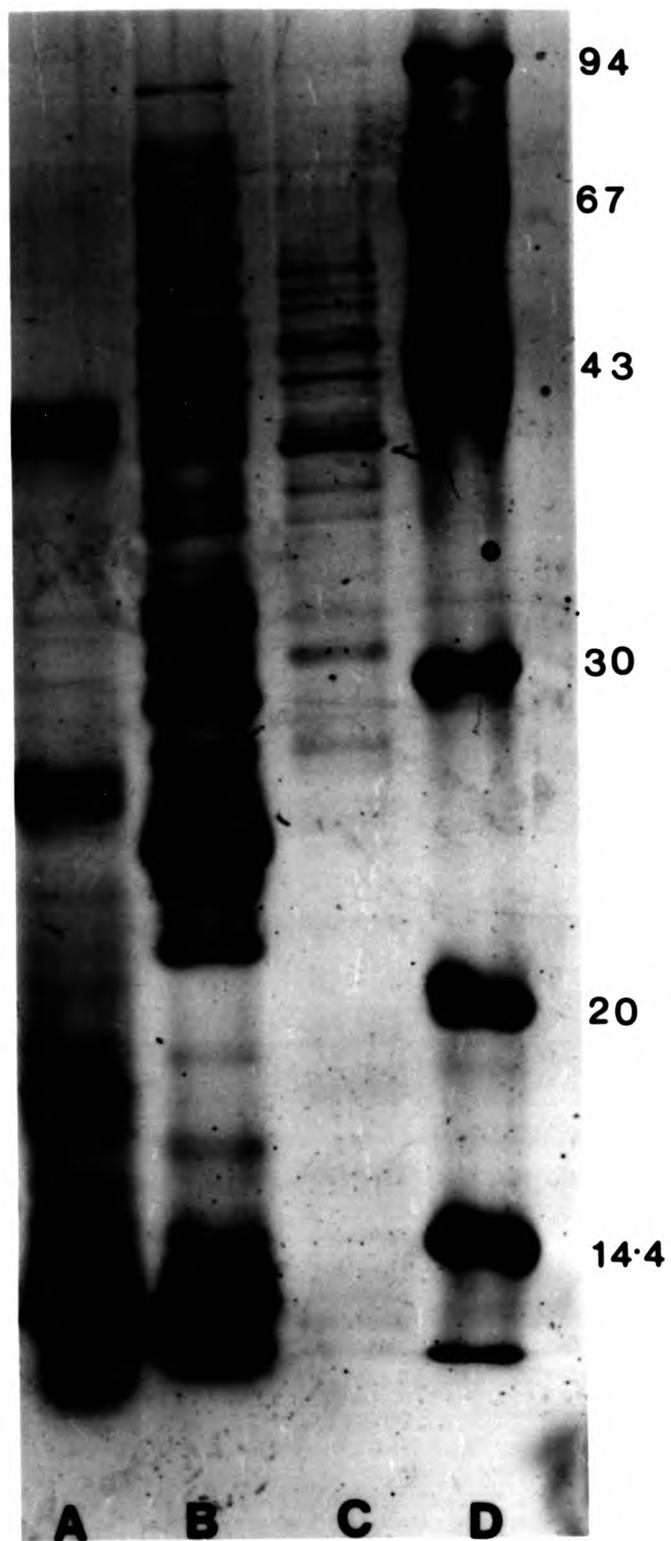
There have been reports of the production of antibodies to M proteins by pepsin extracts of Lancefield group A streptococci injected in an oil based adjuvant, such as Freund's complete adjuvant (Cunningham and Beachey 1974). Rabbits were immunised with an initial dose (0.8mg) of pepsin extract emulsified in Freund's complete adjuvant. The rabbits were boosted two weeks later, using the same intramuscular route with a second 0.8mg dose of vaccine. The intramuscular route was chosen because of the viscosity of the vaccine emulsion. Intravenous inoculation would have been impossible and the subcutaneous route was avoided because this adjuvant could have produced severe ulceration of the skin at the site of inoculation. Sera obtained at 2, 4, 6, 8 and 12 weeks after immunisation were tested for opsonic and precipitin activity against homologous and heterologous type antigens. Group antibodies were not present. Antibodies to the T protein were

detected as early as week 2 and persisted until week 12 of the inoculation schedule. However, precipitin antibodies to M proteins were first apparent as weak lines in gel diffusion tests at week 8.

Electrophoretic and immunoblot analysis of pepsinised extracts of groups C and G streptococci

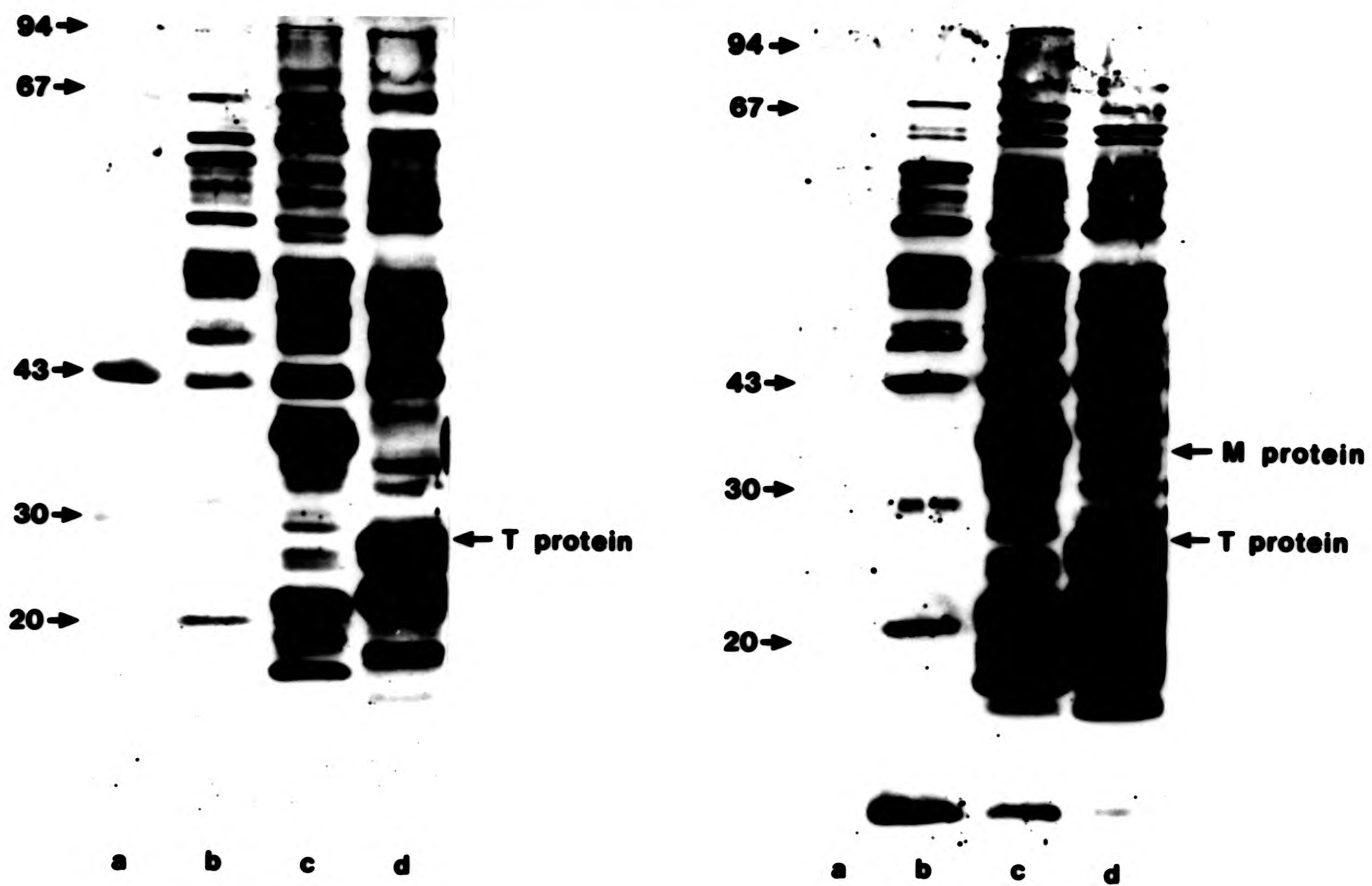
Separation on SDS PAGE gels (12.5%) revealed the numerous distinct bands present in peptic digests of streptococci. Fig. 23 shows the protein patterns obtained before and after treatment with pepsin. Numerous protein bands were present within the molecular weight range of approximately 20,000 to 67,000 daltons. Immunoblot localisation experiments were performed on a representative strain of each of the two groups C and G. The approximate location of the T and M proteins were determined for group C type 204 and group G type 308 using polyclonal rabbit antisera specifically prepared against the T protein (I) and an antiserum prepared using whole cell vaccines (II) known to contain both T and M protein antibodies. A modified ELISA was performed on the nitrocellulose protein transfers using alkaline phosphatase labelling. This was found to be an extremely sensitive method. Homologous and heterologous extracts were also tested. Fig. 24 shows the 'ELISA' blots obtained after incubation with antibodies to trypsinised cells of serotype 204 (I) as the primary antibody in the first experiment and against a whole cell antibody preparation of 204 (II) in the second experiment. The immunoblot from (I) clearly indicated an additional band in the profile for C204 not

Fig.23 SDS-PAGE of pepsin extracted Lancefield group G streptococci



A) pepsin enzyme, B) pepsinised streptococci, C) untreated streptococcal cells, D) Low molecular weight markers $x10^3$

Fig.24 Localisation of streptococcal cell wall proteins by immunoblotting



a) LMW markers, b) undigested streptococcal cells, c) pepsin digest of G308, d) pepsin digest C204

present in the profile for the heterologous extract. The primary antibody used was anti-T for serotype 204, thus the additional band present in C204 pepsin may be the T protein with an approximate molecular weight of 27,000 daltons. This is in accord with previous results obtained (see Section 3.3.7.). In the second immunoblot, where the primary antiserum possessed both M and T-protein antibodies, two additional bands were apparent in the profile for C204. One band was in the molecular weight region of 27,000 (possibly the T protein) and the second band was within the molecular weight region of approximately 34,000 daltons. This is very close to the estimated molecular weight determined for Lancefield group A streptococcal M proteins, extracted with pepsin, which are said to have a molecular weight of 33,500 daltons (Beachey et al. 1977). Densitometry tracings confirmed these findings.

Summary

M protein was extracted from Lancefield group C and group G streptococci with pepsin at pH 5.8. It was found to be highly immunogenic and opsonic antibody titres of 1 in 2 developed after injection of 2mg doses of the pepsinised extract emulsified in Freund's complete adjuvant. The antisera produced single precipitin lines in gel diffusion tests against crude HCl extracts of the homologous M protein. Double lines indicative of the presence of non type-specific antibodies were absent when pepsinised vaccines were used.

3.4.4. OPSONISATION OF STREPTOCOCCI WITH TYPE SPECIFIC HOMOLOGOUS ANTIBODIES

Antisera which had been prepared previously against whole cells of streptococci (which had the capability to survive and multiply in normal human blood) were examined for their opsonic activity in the bactericidal neutralisation test. Ideally, when the homologous type antibody is added to a bactericidal system the streptococci are opsonised and killed by phagocytosis. Fourteen antisera which had produced excellent precipitin lines in gel diffusion against their homologous crude acid extracts of group C and group G streptococci were tested for their ability to opsonise streptococci of the homologous type (Table 44). The results illustrated that all the antisera (with the exception of C308 and perhaps C305) exhibited a remarkable consistency in their degree of opsonic activity. Without the presence of type-specific antibody in the system, the streptococci survived and multiplied in the donors' blood. When the homologous antibody was added to the system the streptococci were opsonised and killed by phagocytosis (Fig. 25). Results from both donors were consistent with the exception of the results obtained with the C305 serum. When C305 antiserum was added to the bactericidal system, an opsonisation effect was seen only with the blood from donor A, the streptococci being opsonised and killed by phagocytosis. With the blood from donor B, the presence of homologous antibody in the system did not prevent the growth of the organisms. The antiserum prepared against an extract of a strain of group C type 308 did not exhibit type specific antibody. There was no bactericidal action with this

Table 44

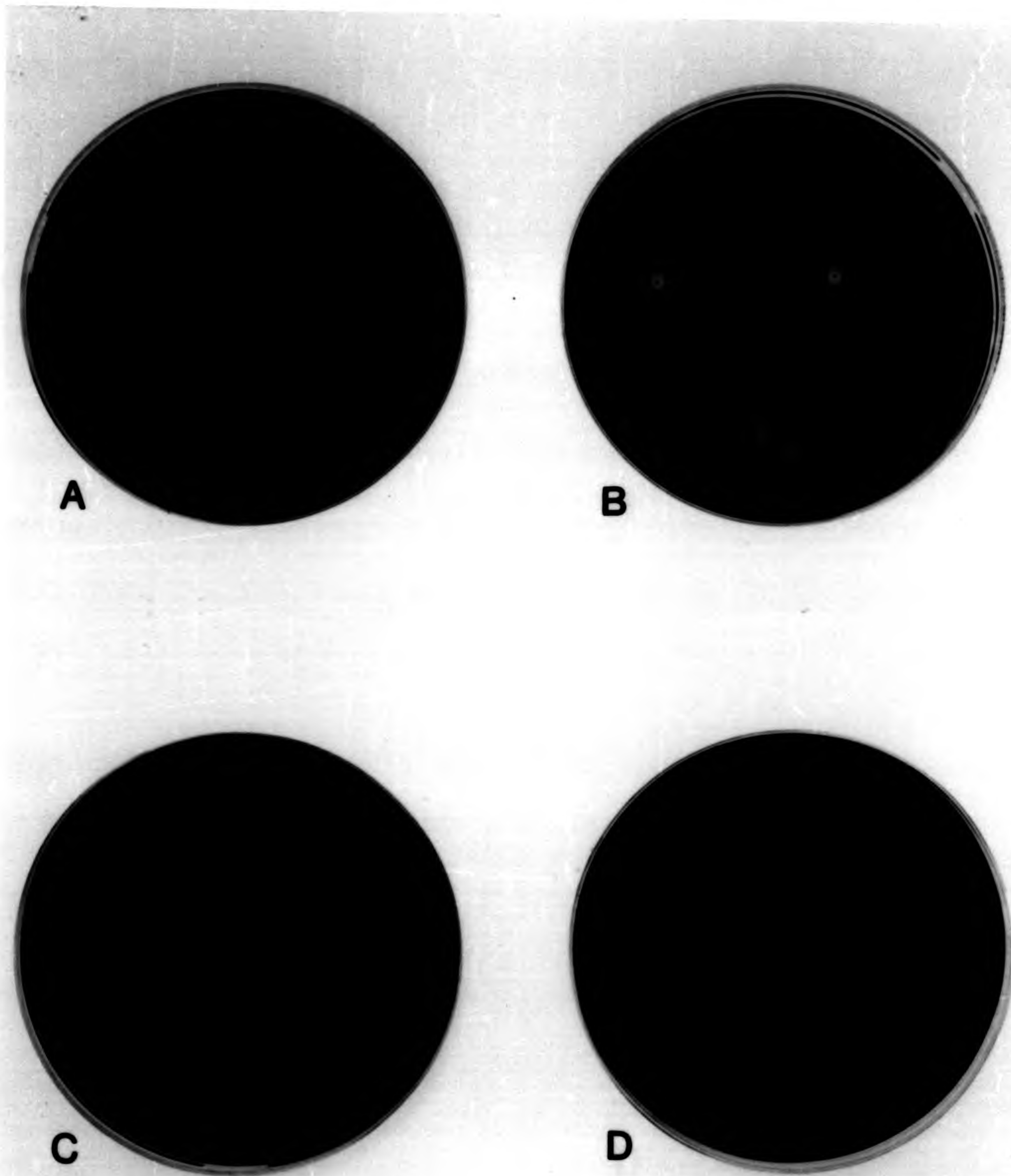
Indirect bactericidal neutralisation test in normal human blood

Strain	Bacterial count	No. of surviving colonies				
		Donor A		Donor B		
		*Homol.	†Heterol.	Homol.	Heterol.	‡NRS
G 16	180	3	UC	15	C	UC
G 21	185	10	UC	12	SC	UC
G 300	220	70	UC	9	SC	UC
G 301	170	12	C	65	C	C
G 302	192	9	UC	50	UC	UC
G 303	200	60	UC	30	UC	UC
G 308	228	4	SC	25	UC	UC
C 7	200	20	12	0	30	28
C 20	150	1	152	22	200	190
C 21	184	7	UC	14	100	UC
C 202	230	0	250	6	150	245
C 204	173	50	UC	25	SC	UC
C 305	196	60	UC	UC	SC	UC
C 308	231	6	50	29	60	65

C: Confluent bacterial growth
SC: Semi-confluent
UC: Uncountable

* Culture plus homologous antiserum
† " " heterologous "
‡ " " normal rabbit serum

Fig.25 Bactericidal neutralisation of Lancefield group C and group G streptococci with type specific antisera



A & C: Survival and multiplication in normal human blood of a group C and group G strain, respectively

B & D: Addition of homologous type specific antibody to the system

serum even though it exhibited precipitin properties in immunodiffusion. It may be that the animal responded to an M-protein moeity which exhibited precipitin properties but was not protective. It has been shown with group A streptococci that certain components of the M-protein molecule induce antibody which exhibits precipitin properties but not bactericidal properties (Fischetti et al. 1976). With this exception, the samples tested exhibited the bactericidal and precipitin properties indicative of M antisera.

Overall, it seemed that isolates of group C and group G streptococci from blood cultures demonstrated a greater degree of virulence by their survival in normal human blood and immunogenicity in rabbits than those strains isolated from less challenging infections (Fig. 26). Fig. 26 also illustrates the variability in the degree of survival and virulence of streptococci with identical serotype antigens. The streptococci of serotype 16 isolated from the blood and throat of different patients both demonstrated M-precipitin activity in gel diffusion against the homologous opsonic antiserum for type 16 (Fig. 27). However, their survival in blood was not the same. This was also demonstrated by strains of serotype 308 isolated from perineal isolates and blood cultures. This phenomenon was demonstrated with other different serotypes which possessed M-precipitin activity but exhibited variability from strain to strain in their survival in blood and their immunogenicity in rabbits. Strains of group C or G streptococci isolated from carriers (predominantly

Fig. 26 Survival of Lancefield group G streptococci in normal human blood.

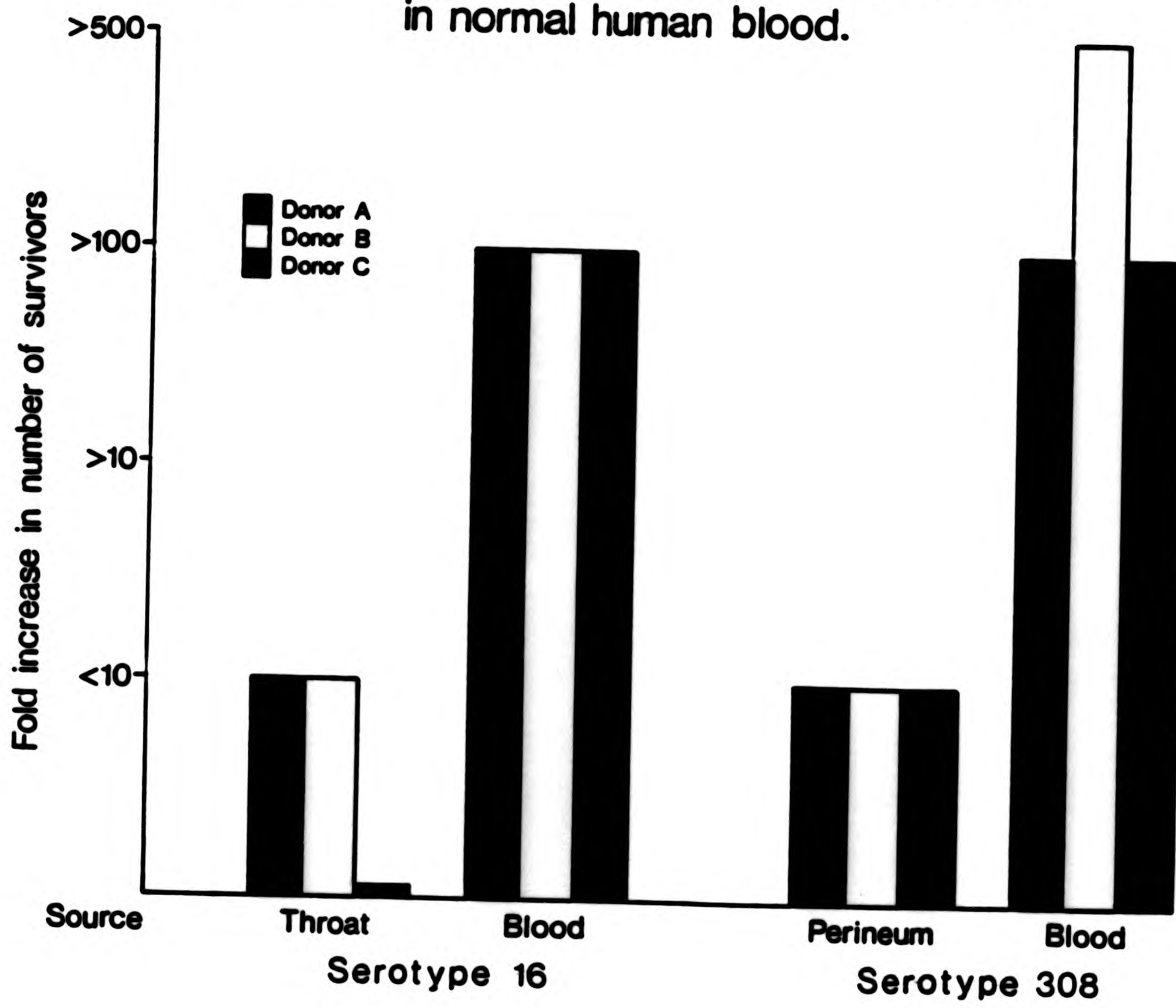
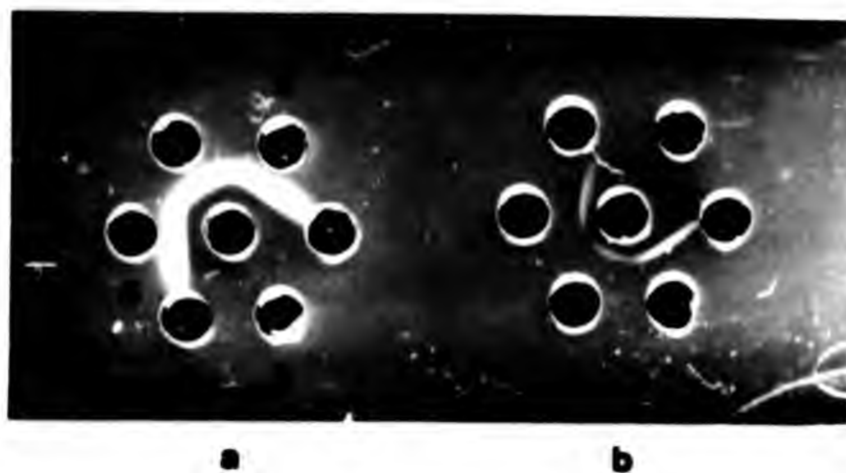


Fig. 27 Immunodiffusion of group G streptococcal M proteins



a: Centre well, antiserum to M-type 16

b: Centre well, antiserum to M-type 308

Peripheral wells contain hot-acid extracts of group G streptococci:

1. Type 16 (Blood) 2. Type 16 (Throat)

3. Type 308 (Blood) 4. Type 308 (Perineum)

5. Control homologous antigen extracts

Antisera were raised against whole heat-killed cells and absorbed with a stock strain of group G streptococcus to remove group-specific antibodies.

throat carriage) did not initially survive in blood and were not immunogenic in rabbits. However, strains isolated from septicaemia or serious systemic infections exhibited greater uniformity in their survival and immunogenicity.

3.4.5. APPLICATION OF THE OPSONIC ANTISERA TO CLINICAL ISOLATES FROM SERIOUS INFECTIONS

Crude acid extracts were prepared from strains of group C and group G streptococci isolated from serious diseases, and tested in double gel diffusion for the presence of M proteins. Extracts were prepared from all strains submitted to the Streptococcus Laboratory for typing during 1986. A group of isolates from minor infections and carriers were also tested for the presence of M-protein antigens. Approximately 12% of Lancefield group C and group G streptococci received for typing during 1986 were from blood cultures. The majority of blood culture isolates exhibited both precipitin properties against homologous opsonic antisera and survival in blood, whereas strains isolated from mild infections (for example, leg ulcers) exhibited variability from strain to strain in their ability to survive in blood. This was illustrated by strains collected from a series of patients (Table 45). Strains originally isolated from superficial sites such as colonisation of the perineum of patients in a maternity ward appeared to be deficient of the anti-phagocytic factor for their survival in blood. However, the M protein may not be the only determinant of virulence in these organisms.

Table 45

The application of opsonic antisera to clinical isolates of Lancefield group C and group G streptococci

	Disease	Source	Lanc. group	M type	Survival in blood
	Septicaemia	blood	G	N.T.	+
	" + osteomyelitis	"	"	N.T.	
	Septicaemia	blood	G	16	+
	" + Ca prostate	"	"	"	
	" + Ca breast	"	"	16	
	" CVP line infection	blood	G	21	+
*	" wound infection	blood	G	202	+
	" osteomalacia of humerus	blood	G	300	+
	" chronic osteomyelitis	"	"	"	
	" fatal anaemia and pyrexia	blood	G	7/302	+
*	" leg sores	"	G	"	
	" cellulitis of leg	"	"	"	
	" P.U.O.	"	"	"	
	Septicaemia	blood	G	305	+
	"	"	"	"	
	" dental abscess	"	"	"	
*	" + sputum isolate	"	"	"	
	Septicaemia	blood	G	308	+
	" septic arthritis	"	"	"	
	" " "	"	"	"	
	Septicaemia	"	C	305	+
*	"	"	"	"	
*	"	"	"	"	

Table 45 (continued)

Septicaemia	Blood	C	301	+
" septic arthritis	"	"	"	
Burns infections	Raw areas	G	308	+
Wound infections	Wounds	G	308	-
Wound infections	Leg ulcers	G	308	-/+
Perineal colonisation (maternity unit)	HVS	G	7/302	-
	MSU	"	"	
	Bath	"	"	
	Bidets	"	"	
	Toilet	"	"	
Staff member	Throat	"	307	-
Tonsillitis	Throat	C	204	+
Nephritis	-	"	"	+
Carrier	Throat	"	"	+
AGN	Throat	G	N.T.	+/-
"	Skin	"	"	+/-

* Group C or group G streptococci isolated from other sources in addition to the blood.

+/-: Variability in survival in normal human blood.
-: No survival in blood.

Type-specificity of the M antisera

The M antisera produced appeared to be type-specific with few exceptions. They were tested against a collection of Lancefield group G streptococci isolated from the blood cultures of drug addicts in the U.S.A. (Table 46). The cultures were submitted to our laboratory for serotyping by Professor A.L.Bisno of the University of Tennessee, Memphis, U.S.A. T-protein antigens were first identified and the strains were then tested with the available M antisera for the group C and group G streptococci. Table 46 shows the serotyping results obtained. An M antigen could be identified in four of the strains. However, an M antiserum to group G strains carrying the 4 T antigen was not available. The strains with the complex T pattern 20/300 produced unexpected results with the M sera reacting with three of the antisera in double gel diffusion. The T-antigen pattern 20/300 is uncommon in strains from the U.K. and is distinct from those strains carrying solely the 20T or 300T antigen. Strains that carry either a T-type 20 or T-type 300 antigen possess their own specific M antigen and M-typing sera for these strains did not cross react with the T-type 20/300 strains. It is likely that strains with the complex T-pattern 20/300 may be a new M type. The other cross reaction observed was with group C strains carrying either the 305T or 308T antigen. When screened against M sera for types 305 and 308 precipitin lines occurred against both. Special absorption procedures would perhaps limit the reactions to a single typing serum. These were the only cross-reactions encountered. There was no cross reactivity between acid extracts of stock

Table 46

Serotyping of blood culture isolates of Lancefield group G streptococci from parenteral drug abusers in the U.S.A.

Strain no.	T type	M type
BC 1618/5156	301	301
BC 1434/5157	7/302	302
BC 1714/5158	20/300	16/308/202
BC 1737/5159	4	-
BC 1750/5160	4	-
BC 1454/5161	16	16
BC 1395/5162	20/300	16/308/202
BC 3125/5163	301	301

strains of Lancefield group A streptococci of all recognised M types and the M typing sera produced against group C and group G strains.

Summary

The M protein is an antiphagocytic molecule and is known to be responsible for one form of type specificity. Streptococcal M proteins have been identified in Lancefield groups A, C and G streptococci. The survival and multiplication of streptococci in normal human blood is a direct indication of the presence of M-protein antigens. From the bactericidal experiments it would appear that blood culture isolates of group C and group G streptococci were more likely to be able to survive in normal human blood and were more immunogenic in rabbits than strains from superficial sites. When the homologous type antibody was added to the system the streptococci that would have multiplied in its absence were opsonised and killed. Amongst strains of Lancefield group C and group G streptococci of human origin fourteen distinct M-type protein antigens have so far been identified.

3.5. THE EPIDEMIOLOGY OF LANCEFIELD GROUP C AND GROUP G STREPTOCOCCAL INFECTIONS IN MAN

Comparatively very little work has been done on the typing of human isolates of streptococci of Lancefield group C and group G. Therefore, very little is known of the epidemiology of these

organisms and their ability to cause infectious disease. A collection of 1777 strains belonging to either Lancefield group C or group G was made. These included strains from systemic diseases and clusters of strains from what appeared to be hospital outbreaks. A provisional typing scheme based on the cell wall T-protein antigens was developed in this study to test whether or not these organisms were epidemiologically related and also, if there could be an association between a particular systemic disease and the serotype of the strain responsible.

3.5.1. LANCEFIELD GROUP C STREPTOCOCCI (S.EQUISIMILIS)

A total of 566 isolates of Lancefield group C streptococci (S.equisimilis) of human origin were examined. This total included all group C streptococci isolated from both presumptive outbreaks and apparently random incidents of infection. Overall it was possible to subdivide these streptococci into 14 different serotypes (Fig. 28). There were 103 strains which carried T antigens first recognised in strains of Lancefield group G. These were serotypes 301, 305 and 306 which constituted approximately 18% of the total distribution of serotypes. Serotypes which represented more than 10% of the total were numbered 21, 203, 204 and 301. Together these types formed 59% of the total. Serotype, 204, alone represented 29% of all isolates. The streptococci were recovered from a wide range of sites. Most were from the blood, throat, skin, ulcers, wounds, raw areas and high vaginal swabs (HVS). Few isolates were examined from aspirates, pus, cerebro-spinal fluid (CSF), sputum, the ear, eye, nose,

Fig. 28 Serological subdivision of Lancefield group C streptococci by T-typing over a four year period 1982-1985

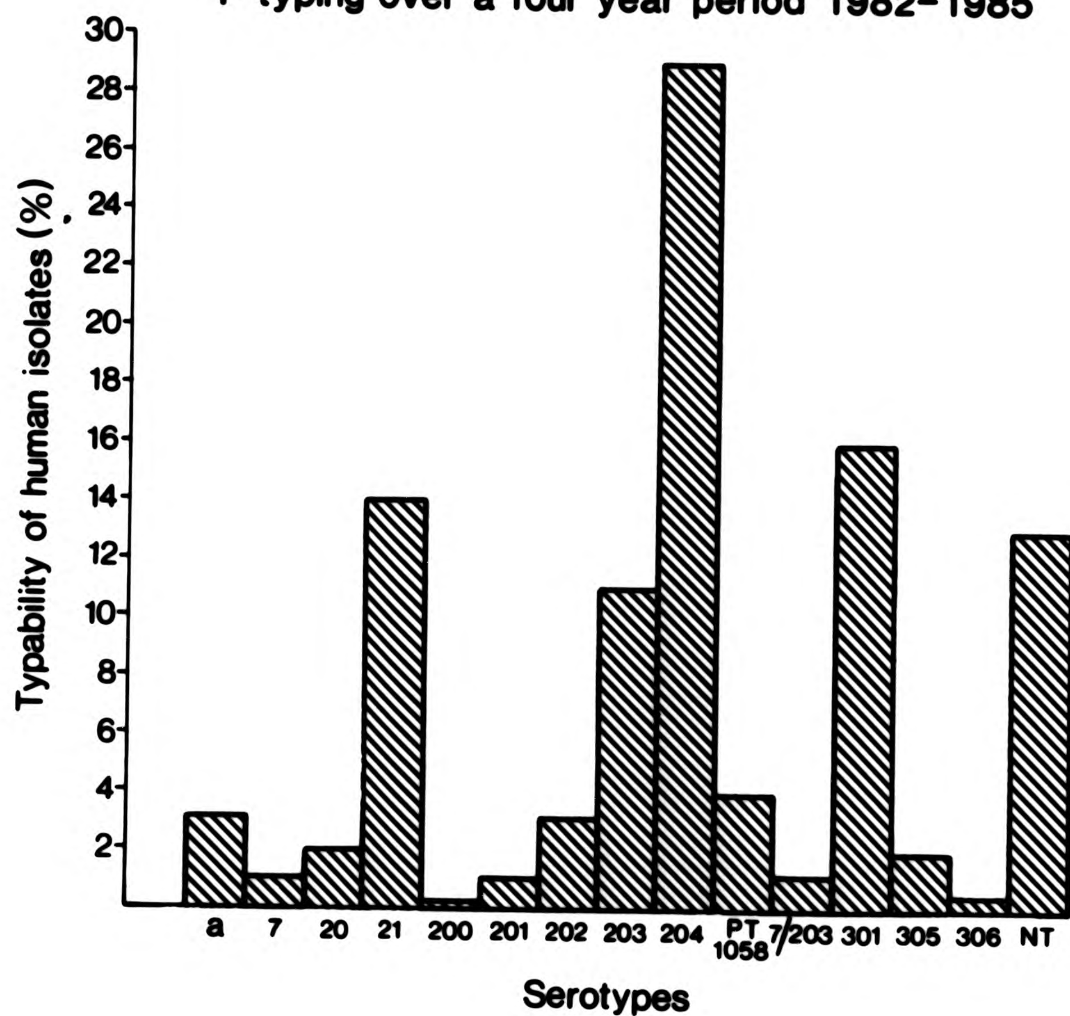
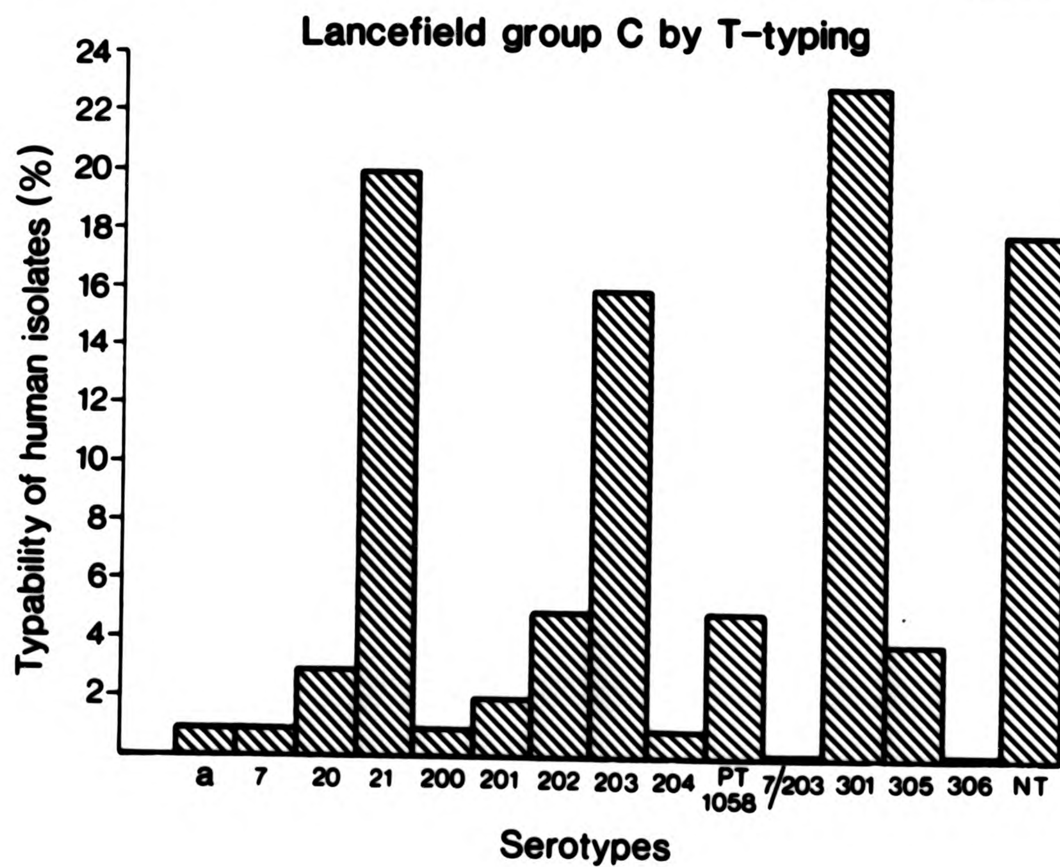


Fig. 29 Serotype distribution of single human isolates of S.equisimilis Lancefield group C by T-typing



umbilicus and urinary tract.

<u>Type distribution among random isolates of Lancefield group C</u>

These were single non-related isolates with 'outbreak' strains being designated as one distinct strain per outbreak. These isolates were divided into 16 different serotypes with a typability rate of 82% (Fig. 29). Table 47 lists the serotype distribution for a total of 380 strains. One hundred and three carried T antigens belonging to Lancefield group G (see previous section) and 6 strains carried T antigens belonging to Lancefield group A (which formed 1% of the total). Serotypes 21 and 301 alone represented more than 20% of the total. The other serotypes produced distribution patterns within the range of 1 to 16%. The most common serotypes were 301, 202, 203, 21 and PT1058. The isolates examined were from a wide variety of different sites which included the throat (39%), leg ulcers (12%), skin (9%), blood (6%), raw areas (6%) and the female genital tract (6%) (Table 47). The most common serotypes found in those patients with sore throat were serotypes 21 and 203. The second largest group of isolates was from leg ulcers with serotype 301 predominating.

Serotypes were evenly distributed among strains isolated from blood cultures. In the main these were from cases of septicaemia and endocarditis. There were four separate incidents of acute nephritis, with both blood culture and throat isolates carrying the same serotype. Two cases were non-typable. One patient had post natal renal failure with the group C streptococcus being isolated

Table 47

Serotype distribution of single human isolates of S.equisimilis (Lancefield group C)

Source	No. of isolates	T serotypes 7	20	21	200	201	202	203	204	PT 1058	7/ 203	301	305	306	28	IMP 19	4	NT
Aspirates	6	.	.	1	.	.	.	2	.	.	.	.	.	.	.	.	.	3
Blood	23	.	.	5	.	.	3	2	.	.	1	7	.	.	.	.	.	5
CSF	3	.	.	.	.	.	.	.	.	.	.	.	3	.	.	.	.	0
Ear	7	.	1	1	.	.	.	1	.	.	.	3	.	.	.	1	.	0
Eye	1	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	0
HVS	24	.	1	2	.	2	2	1	.	.	.	9	.	.	1	.	.	6
Nose	3	.	.	1	.	.	.	.	.	.	.	1	.	.	.	.	.	1
Pus	3	.	1	.	.	.	.	.	.	.	.	1	.	.	.	.	.	1
Raw areas	23	.	.	8	.	.	.	5	.	.	.	.	6	.	.	.	.	4
Sinus	4	.	.	2	.	.	.	.	.	.	.	2	.	.	.	.	.	0
Skin	35	.	1	7	.	.	3	4	.	1	.	12	1	.	.	.	.	6
Sputum	4	.	.	3	1	.	.	.	.	.	.	.	.	.	.	.	.	0
Throat	149	2	5	38	.	3	3	38	5	8	.	13	2	.	.	1	1	30
Ulcers	44	.	.	2	1	.	1	4	.	8	.	20	.	.	1	.	.	7
Umbilicus	3	.	.	.	.	.	.	.	.	.	.	3	.	.	.	.	.	0
Wounds	20	.	1	.	.	.	3	3	.	1	.	6	.	1	.	1	.	4
Other	28	.	.	6	.	1	3	.	.	2	.	11	1	.	.	.	.	4
Total	380	2	10	76	2	6	18	60	5	20	1	88	14	1	2	3	1	71

PT = Provisional type number

from the HVS and blood culture, and, the other developed nephritis two weeks after a sore throat from which a Lancefield group C streptococcus had been isolated. Three cultures only were from patients with meningitis but among the 23 strains from blood cultures ten were from patients with bacterial endocarditis. Four of these last had mitral valve replacements.

Outbreaks of infection caused by Lancefield group C streptococci

Outbreaks due to these organisms do occur. In this study there were six outbreaks of sore throat, presumably due to group C streptococci. Representatives of a single serotype were isolated from a single institution. These outbreaks included episodes of tonsillitis at two schools with T-types PT1058 and type 21 being isolated separately. The third outbreak was of pharyngitis in a military camp with T-type 4 being the serotype isolated from five throat swabs. There were also three incidents of hospital infection: two small and one large outbreak. The first incident of cross-infection occurred on a hospital ward, where the same serotype, type 301 was isolated from the discharging lesions on one patient and from a hip sinus of a second patient. The second incident occurred in a ward where four patients developed skin lesions infected with group C streptococci. The serotype isolated was PT1058.

The largest single outbreak studied occurred during the summer of 1983 at a district hospital in England. This was a unique outbreak of pharyngitis where food was implicated as the vehicle of

transmission. It was confined exclusively to the female staff who had eaten salads. All salads contained sliced hard-boiled eggs. Two members of the kitchen staff admitted to having had sore throats before the outbreak. Throat swabs from them, taken after the outbreak yielded group C streptococci. Subsequently, 146 cases of sore throat emerged amongst the staff and all yielded group C streptococci on swabbing. All cultures belonged to the serotype 204 and were present only in throat swabs. None was isolated from nose swabs. The patients responded promptly to treatment with penicillin or ampicillin. Some were given erythromycin.

Strains for serotyping were also received from an outbreak of pharyngitis which was followed by nephritis in school children in Romania (Mihalcu et al. 1982). These were strains isolated from school children in one particular area that was considered a high risk community for streptococcal infections. Prior to serotyping it was not possible to distinguish between these strains of S.equisimilis. This outbreak occurred in 1970 and the cultures were re-examined with the newly developed typing method. Representatives isolated from patients with pharyngitis or nephritis were examined. Strains from patients with sore throats, nephritis, or asymptomatic carriers were all, bar one, of T-type 204. The exception was a single strain of type 21. Phage-typing and DNA fingerprinting (S.Skjold, Minnesota, U.S.A.) confirmed the similarity of these strains.

Examination of the histograms (Fig. 28 and Fig. 29) illustrating the type distribution amongst human isolates of Lancefield group C demonstrates effectively that the largest single outbreak caused by these organisms was due to serotype 204 (Fig. 28). The graph describing the distribution patterns amongst single isolates included all representatives from outbreaks as one single isolate (Fig. 29). Fig. 28 includes each strain received for serotyping over the four year period.

Human infections caused by other species of group C streptococci

Human infections caused by species of group C streptococci other than *S.equisimilis* are rare. There have however, been recent reports of community outbreaks due to *Streptococcus zooepidemicus*. This organism causes infection in a wide range of animals but it has been rarely found in man (Parker 1983). The few reports of human infection include upper respiratory tract infection, pneumonia, septicaemia, endocarditis and meningitis, usually in patients who have consumed unpasteurised milk or those in close contact with horses (Barnham *et al*. 1983). The outbreaks as opposed to single cases of infection involved the consumption of unpasteurised dairy products. They usually have given rise to pharyngitis with the complication of poststreptococcal glomerulonephritis developing in some patients (Duca *et al*. 1969, Barnham *et al*. 1983, 1987).

A collaborative study was carried out with Dr Michael Barnham of Friarage Hospital, Northallerton; Dr John Tagg from the University

Table 48

Characterisation of strains of *S.zooepidemicus*

Origin	No of isolates	Source
Sporadic infections	9	7: Patients 2: Horses
Outbreak A (U.K.)	16	11: Patients 3: Cows 1: Horse 1: Bulk milk
Outbreak B (New Mexico, MMWR, 1983)	10	8: Patients 1: Cheese 1: Milk
Outbreak C (U.K.) (Barnham *et al.* 1983)	4	4: Patients
Outbreak D (Romania, Duca *et al.* 1969)	2	2: Patients

Investigations:	
Biotyping	CPHL London
T-antigen detection	CPHL London
Bacteriocin typing	New Zealand
Bacteriophage typing	Minnesota, U.S.A.
Antibiotic sensitivity	Northallerton

Table 49

Typing of S.zooepidemicus isolates

Isolate no.	VP	HIP	ESC	PYR	AGL	GUR	BGL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLY	*BHS	+API profile no.
SPORADIC INFECTIONS																						
1.	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	4463607
2.	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	4463607
3.	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	4463617
4.	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	4463607
5.	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	4463607
6.a-c	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	4463607
7.	-	-	+	-	-	+	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	4461617
HALIFAX OUTBREAK (A)																						
8.a-o	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	4463607
8.p	-	-	+	-	-	+	-	+	+	+	-	-	-	+	+	-	-	-	+	+	+	4461607
NEW MEXICO OUTBREAK (B)																						
9.a-j	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	4463607
NORTHALLERTON OUTBREAK (C)																						
10.a-c	-	-	-	-	-	+	+	+	+	+	-	-	-	+	+	-	-	-	+	+	+	0471607
10.d	-	-	+	-	-	+	-	+	+	+	-	-	-	+	+	-	-	-	+	+	+	4461607
ROMANIA OUTBREAK (D)																						
11.a	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	4463607
11.b	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	+	+	+	+	4463647
VETERINARY INFECTIONS																						
12.	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	4463607
13.a-d	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	4463607

* BHS: beta haemolysis
\+ Component tests of API kit listed in Appendix 1

Combined results of typing

Bacteriocin	:	Bacteriophage	:	API Profile	Isolate numbers showing this pattern
P000, S53	:	12	:	4463607	4, 12, 13a, c,
P000, S53	:	2/3/4/6/12/14	:	4463607	9 a-f, i,
P226, S53	:	7/11/12/13	:	4463607	9g, h,
P226, S57	:	2/7/11/12	:	4463607	8a-g, i-1, o,
P000, S57	:	12	:	variable	3, 10b-d,

Table 50

Failure to detect T antigens amongst species of Lancefield group C streptococci other than human isolates of S.equisimilis

Species	No. isolated	T type
S.dysgalactiae	5	-
S.equi	6	-
S.zooepidemicus	50	-
S.equisimilis	8	-
† S.milleri	24	-

All isolates from animals, except those of S.milleri.

† Isolates of S.milleri with the Lancefield group C polysaccharide antigen.

of Otago, New Zealand and Dr Stephen Skjold from the University Medical School, Minneapolis, Minnesota, U.S.A. An international collection of strains of S.zooepidemicus from human infections and also strains from animals was formed. The aim of the investigation was to test strains from the U.K. and abroad, to compare the isolates involved and to develop a system to aid epidemiological studies (Barnham et al. 1987). The strains were studied as shown in Table 48. Lancefield grouping and biotyping were performed by the methods previously described. T-protein antigens were sought but not found on any isolate. The results are shown in Table 49. Combining the results by bacteriocin, bacteriophage and the API profile, isolates in the collection could be grouped into five main patterns (Table 49). Other combined patterns were seen with individual isolates. Indistinguishable strains were found within many of the outbreak strains. In all the outbreaks, infections were attributed to consumption of unpasteurised dairy products.

For comparison other animal isolates were tested and it was not possible to detect T-protein antigens on any of them (Table 50).

3.5.2. LANCEFIELD GROUP G STREPTOCOCCI

A total of 1211 isolates of Lancefield group G streptococci were examined during the four year study. On the basis of T-typing it was possible to subdivide these strains into 21 serotypes and the serotype distribution patterns were more evenly distributed than among the Lancefield group C streptococci. Again, there were

instances of cross-over of type antigens. Some 5% of strains carried T antigens of types 2, 4, 28, 8/25/IMP19, ordinarilly associated with Lancefield group A. Some 13% carried one or other of the original Griffith's type antigens 7, 16, 20 or 21 and only 1% reacted with antisera that had been prepared against Lancefield group C in particular serotype 202. None of the serotypes represented more than 20% of the total distribution and those forming more than 10% of the total were serotype, 301 and the complex pattern 7/302. Together these two types made up more than 30% of the total distribution (Fig. 30). However, 18% of the collection could not be typed with the antisera available. The most common serotypes overall were types 301, 7/302, 20, 16 and 308. Five of the serotypes were not recognised until 1983 hence the overall improvement in the typability rate during the four year period described here in comparison with the two year study previously described (Efstratiou 1983). The typability rate overall for the Lancefield group G streptococci was 82%.

Type distribution of random isolates of Lancefield group G

The data brought together in Table 51 show that the group G streptococci isolated as single strains could be subdivided into 22 T-types. Again, there were some instances of cross-over between particular T antigens belonging to Lancefield groups A (6%) and C (14%). These were types 2, 4, 28, 8/25/IMP19 for group A, and Griffith's original types 7, 20 and 21 for group C. Serotype 302 which always occurred as a complex with serotype 7 was the most common single type and represented 18% of the isolates. The

Fig. 30 Serological subdivision of Lancefield group G streptococci by T-typing over a four year period 1982-1985

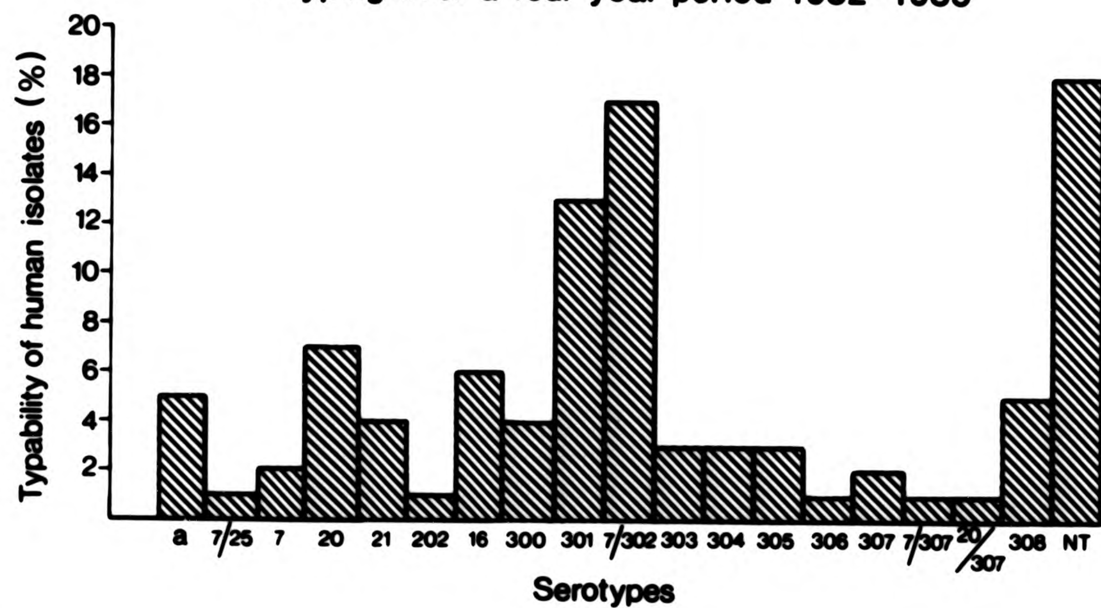


Fig. 31 The typability of single human isolates of Lancefield group G streptococci by T-typing

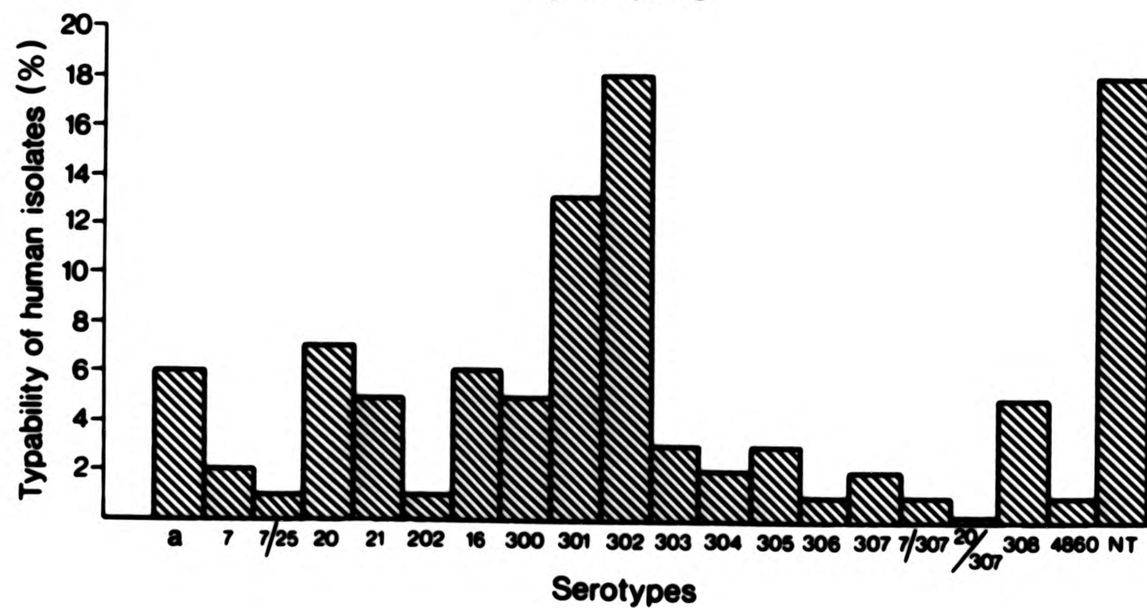


Table 51

Serotype distribution of non-related human isolates of Lancefield group G streptococci

Source	No. of isolates	T serotypes: 16	300	301	7/ 302	303	304	305	306	307	7/ 307	20/ 307	308	7	7/ 25	20	21	4	28	IMP 19	202	PT 4860	2	NT
Aspirates	18	2	.	3	1	.	.	.	.	.	.	.	.	.	.	5	3	.	.	2	1	.	.	1
Blood	69	6	1	10	15	1	2	1	1	2	.	.	2	1	.	5	2	.	3	1	2	1	.	13
CSF	1	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Ear	25	.	2	2	4	.	1	1	.	1	1	.	1	.	.	2	2	.	1	2	.	.	.	5
Eye	12	1	.	2	.	2	.	.	.	.	.	.	.	1	.	1	1	.	1	.	.	.	.	3
Faeces	2	.	.	.	1	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	0
HVS	81	1	3	7	23	.	.	1	.	3	2	.	5	1	1	8	7	.	2	.	2	2	.	13
Nose	7	1	.	.	4	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	1
Pus	9	.	1	4	3	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	0
Raw areas	102	11	4	23	12	3	3	7	2	2	.	1	8	.	.	4	8	1	1	3	.	.	.	9
Skin	109	8	3	21	19	3	6	.	2	.	.	.	7	6	.	10	3	1	2	.	.	.	1	17
Sputum	10	1	.	1	2	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	5
Throat	237	21	23	41	27	1	3	7	6	6	.	.	14	.	.	21	8	.	6	4	2	4	.	43
Ulcers	210	6	6	20	31	14	6	14	.	3	4	.	4	9	.	8	5	.	19	7	.	.	.	53
Umbilicus	7	1	.	2	1	1	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	0
Urinary tract	29	2	2	1	14	1	.	.	.	1	.	.	1	.	.	1	2	.	1	.	.	.	.	3
Wounds	148	4	8	12	33	6	1	3	1	1	5	.	5	8	1	9	7	.	2	7	1	2	.	32
Other	91	6	1	9	18	1	2	6	.	2	.	.	3	1	8	6	3	1	2	2	.	2	3	15
Total	1167	71	54	158	209	33	24	40	12	21	12	1	52	30	10	81	51	4	40	28	8	11	4	213

percentage distribution of the other types was within the range of 1 to 13%. Type 301 or the complex 7/302 were carried by 31% of the strains examined (Fig. 31). The serotypes were generally evenly distributed and the system thus possessed a useful discriminatory power. The largest groups examined were from the throat (20%), leg ulcers (18%), wounds (13%), skin (9%), the female genital tract (9%), the blood (6%) and from infected burns (9%). The remaining isolates (16%) were from aspirates, CSF, pus, the ear, faeces and other sites. Many of these group G strains were isolated from cases of severe diseases including septicaemia, endocarditis and septic arthritis. Cultures were also recovered from cases of tonsillitis, otitis media, impetigo and cellulitis. The most common serotypes were the complex pattern 7/302, 301, 16, 300 and 308. Type 308 was the most recent addition to the set of T-typing antisera. It was not introduced until mid 1983. Some strains were therefore not tested against this antiserum or the other typing sera introduced after 1982. These were serotypes 304 and 307. However, it was noteworthy that the original T-types (16, 7, 20 and 21) first isolated by Griffith in 1934 appeared to be associated particularly with pharyngeal infections. Many different serotypes were isolated from leg ulcers and other skin infections. There were only two non-related isolates of group G from faeces. Isolates from the category described as 'other' in Table 51 included sites such as the rectum, peritoneum, perineum, mouth and sinuses. The typability rates by source were scattered between 74% and 100%.

Serious systemic disease caused by Lancefield group G streptococci

Many random isolates were from cases of severe infections such as septicaemia, endocarditis, meningitis and septic arthritis. Cases of septic arthritis were unexpectedly numerous totalling 14 cases. The most common serotype isolated from one or both of the joint fluid and blood of patients with this condition was serotype 20. Several of these patients presented with conditions such as phlebitis of the leg which eventually gave rise to septic arthritis. Only one of the 14 isolates could not be serotyped. Among these 14 were seven isolates that were included in the clinical study undertaken by Gaunt and Seal (1986). Their seven cases were compared by them to the 29 reported in the literature. Group G streptococcal carriage (perianal) was detected in only one of the seven patients. Cellulitis accompanied group G streptococcal arthritis in all but one of the cases reviewed. There was no association between any particular T-type and joint infection, but group G strains isolated from other sites at the time of infection possessed the same serotype as those from the joints.

There were 69 isolates from the blood and there was a wide distribution of serotypes. Serotypes 16, 301, 7/302 and 20 appeared to be the most common. Many of the blood culture isolates were from patients with septicaemia associated with spontaneous peritonitis, catheter sepsis, infected leg ulcers, cellulitis, bronchial pneumonia and acute renal failure. Blood cultures were also received from cases of endocarditis. Type

associations between serious diseases and serotypes indicated that, three T-types predominated amongst isolates from cases of septicaemia and endocarditis. These were types 16, 301 and the complex pattern 7/302. There were five isolates from blood cultures which had been referred for serotyping from the U.S.A. The clinical features of group G streptococcal bacteraemia in nine patients at a hospital in Nottingham were studied by Finch and Aveline (1984). The organisms isolated belonged to five different serotypes (Table 52). There was no evidence of any link between the patients with identical serotypes. All but two patients had predisposing factors such as cardiac failure or malignant disease. There were two strains only from cases of meningitis: one recovered from CSF the other from blood. There were five instances of blood culture and throat isolates from the same patient. Invariably, the serotypes were identical. Isolation of group G streptococci from faeces appears to be a rare event. In this study, there were two isolates only from such specimens. Both patients had clinical symptoms of severe abdominal pain and bloody diarrhoea. One organism was identified as type 7 and the other carried the complex 7/302. A more common source of group G infections was the middle ear and 25 strains of various serotypes were from this organ.

Strains were available from 89 neonatal or maternal infections. Since group G streptococci may be carried asymptomatically in the female genital tract, occasional neonatal infection is not unexpected. Mother and baby related strains were common. The

Table 52

Case studies of patients with group G streptococcal septicaemia

Patient	Age	Sex	Diagnosis	Portal of entry	T type
1	72	F	Endocarditis	Not known	307
2	77	M	"	"	20
3	63	M	Septicaemia	Infected drip site, cellulitis	20
4	74	M	Endocarditis	Dermatitis	21
5	82	M	Septicaemia	Ulcers, cellulitis	28
6	69	F	"	Wound infection, cellulitis	20
7	59	F	"	Ulcerating breast carcinoma	307
8	82	F	"	Ulcers, cellulitis	7/302
9	69	M	"	Ulcers	308

most common serotypes among these pairs were 7/302, 20, 21, 301 and 308. Serotype 308 was based on strains isolated from one outbreak of post-puerperal infections in a maternity unit. These infections occurred from 6 to 20 days post partum and were usually accompanied by pyrexia. Most commonly group G streptococci were isolated from the HVS of these patients. Offensive lochia were recorded.

As with group C, those group G strains isolated from animals could not be serotyped. There were 14 animal isolates available. All bar one were from urinary tract infections in dogs. The exception was isolated from a cat with pharyngitis.

Outbreaks of infection caused by Lancefield group G streptococci

Strains were available from possible outbreaks of hospital-acquired infection due to group G streptococci that occurred in 30 institutions in the U.K. alone during the four year study period. The main categories of infections in these outbreaks were pharyngitis and skin sepsis but some patients developed septicaemia. The main outbreaks studied may be summarised as:

a) Streptococcal infections in a London burns unit.

b) Outbreaks of skin sepsis in hospital wards.

c) Outbreaks of infection in a London leg ulcer clinic.

d) Group G streptococci in the West Indies.

e) Group G streptococcal infections in maternity units.

f) Asymptomatic carriage in institutions.

g) Foodborne outbreaks of group G streptococcal pharyngitis.

Streptococcal infections in a London burns unit

Outbreaks of infection in burns units caused by pyogenic streptococci is a serious complication. Among such organisms attention has almost invariably been directed towards S.pyogenes. In one burns unit group G streptococci have always been numerous among the streptococci isolated from the raw areas at the sites of the injury. A prospective survey was therefore carried out in this unit. During the survey, a new burns unit was built. The study was undertaken in collaboration with the Burns Unit, Queen Mary's Hospital, Roehampton, under the guidance of Dr M.Perinpanayagam and the assistance of Dr M.Weinbren and Dr G.Calver (who drew attention to this problem).

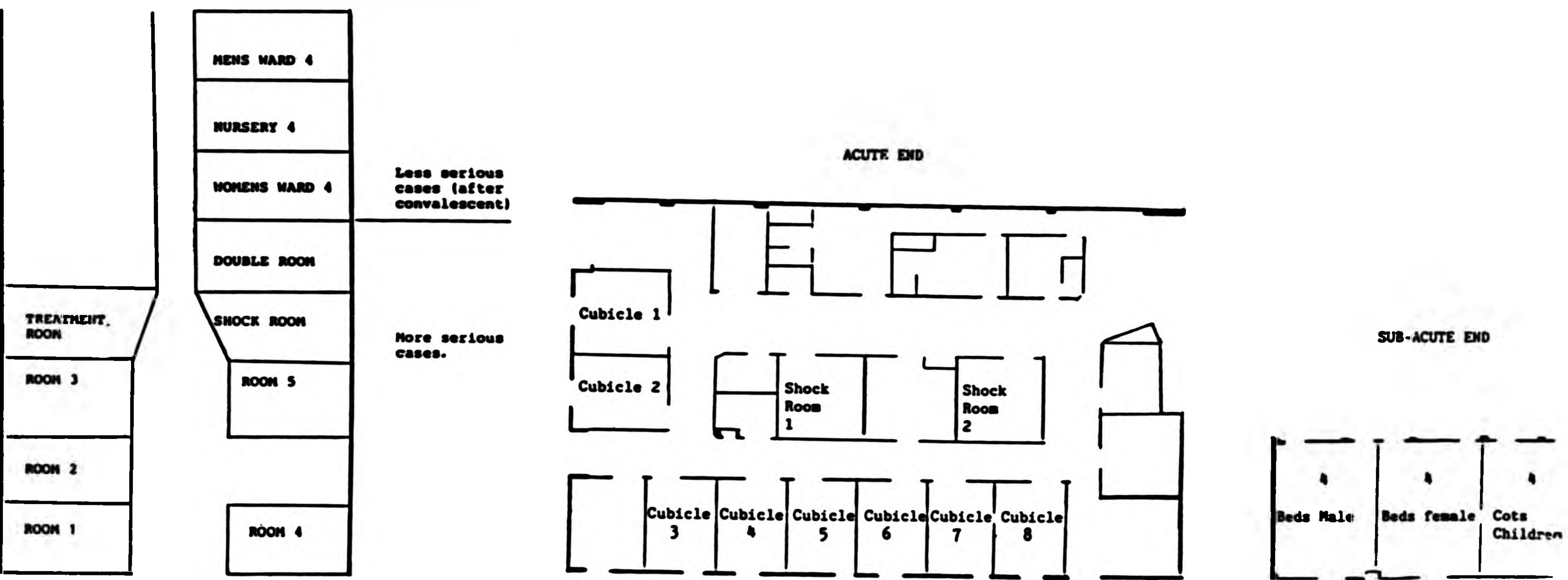
Hospital accomodation

The burns unit serves the whole of the South West Thames health authority. The old unit accommodated 17 patients, five in single cubicles and 12 in open four-bedded wards (Fig. 32). There was no special ventilation system in the old unit. The new burns unit accommodates 20 patients, eight in single cubicles and 12 in open four-bedded wards (Fig. 32). Ducted and filtered air is now provided.

The outbreaks

A total of 759 patients were admitted to the unit during the years 1982 to 1985. The monthly isolations of group A and group G streptococci are shown in Fig. 33. A total of 155 (20%) patients were colonised with group G and 116 (15%) with group A streptococci (Table 53). Both were present in 31 patients (4%). These

Fig.32 Diagrammatic representations of the 'old' and 'new' Burns Unit at QMH London



'Old' Burns Unit

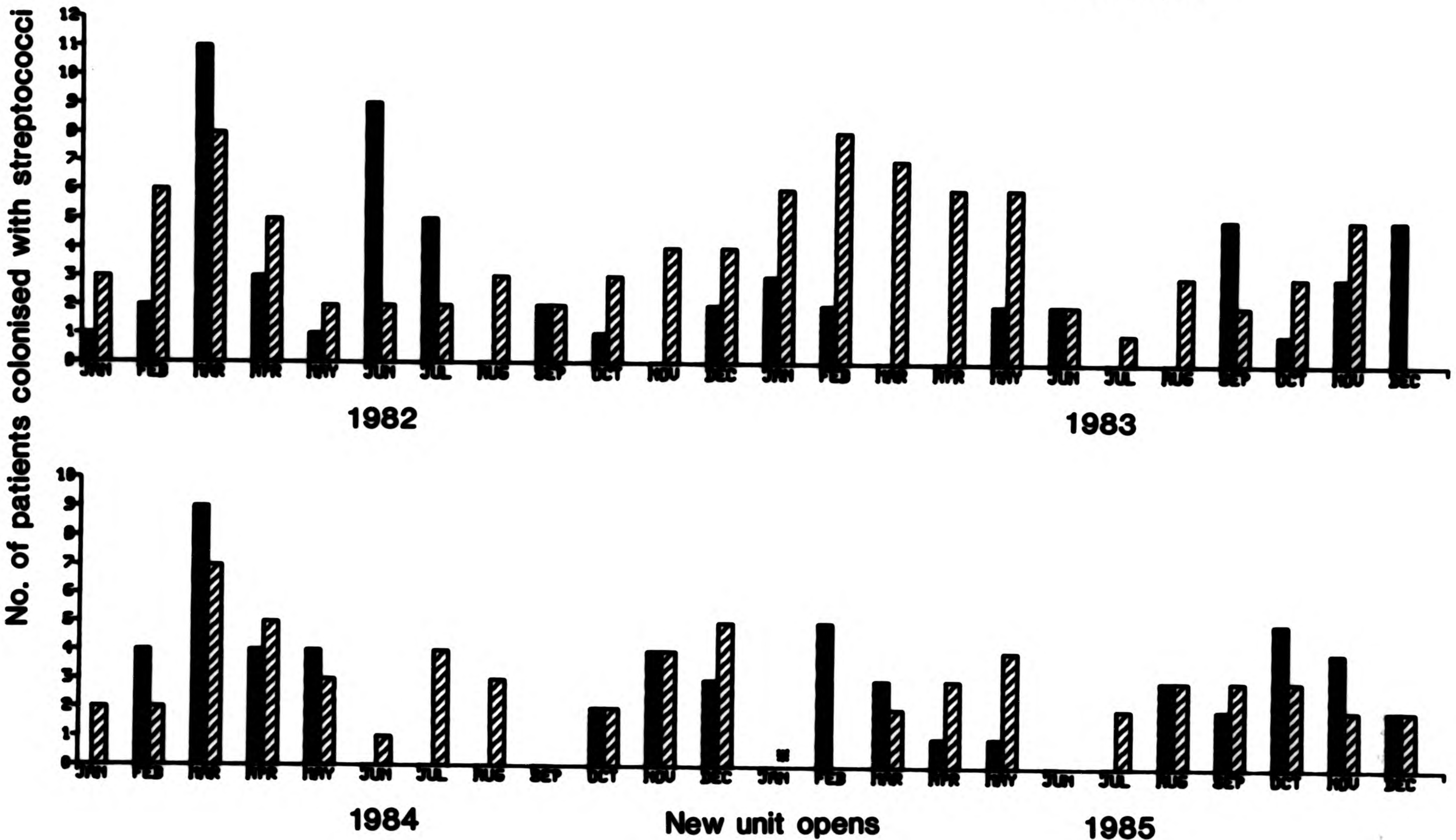
'New' Burns Unit

Table 53

Streptococcal isolations from burns patients

<table>
<tr><th></th><th>1982</th><th>1983</th><th>1984</th><th>1985</th></tr>
<tr><td>Nos. of patients admitted</td><td colspan="2">348</td><td>171</td><td>240</td></tr>
<tr><td>Nos. colonised with group A</td><td>37</td><td>23</td><td>30</td><td>26</td></tr>
<tr><td>Nos. colonised with group G</td><td>44</td><td>49</td><td>38</td><td>24</td></tr>
</table>

Fig. 33 Isolation of Lancefield group A and group G streptococci in a London Burns Unit



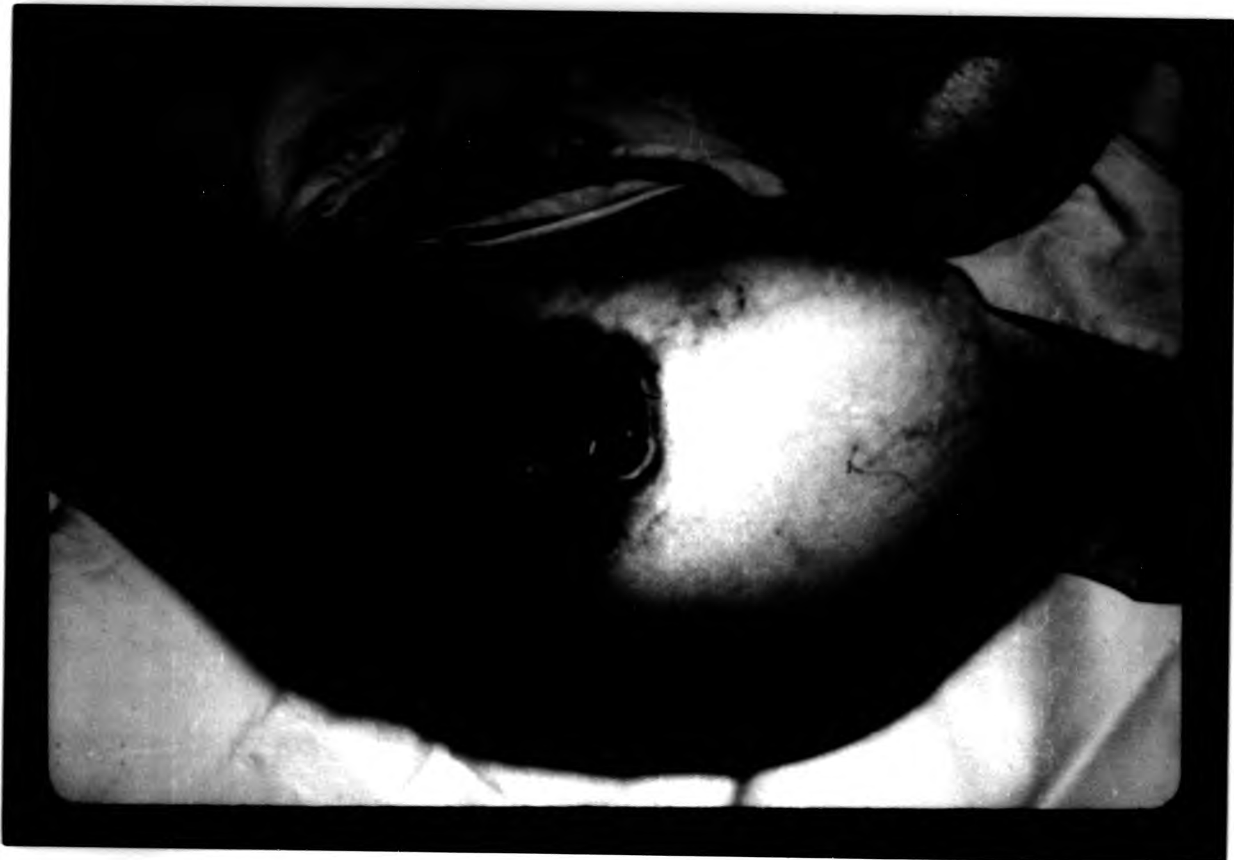
figures are in considerable excess of the 3.7% infection rate given by Lawrence (1985).

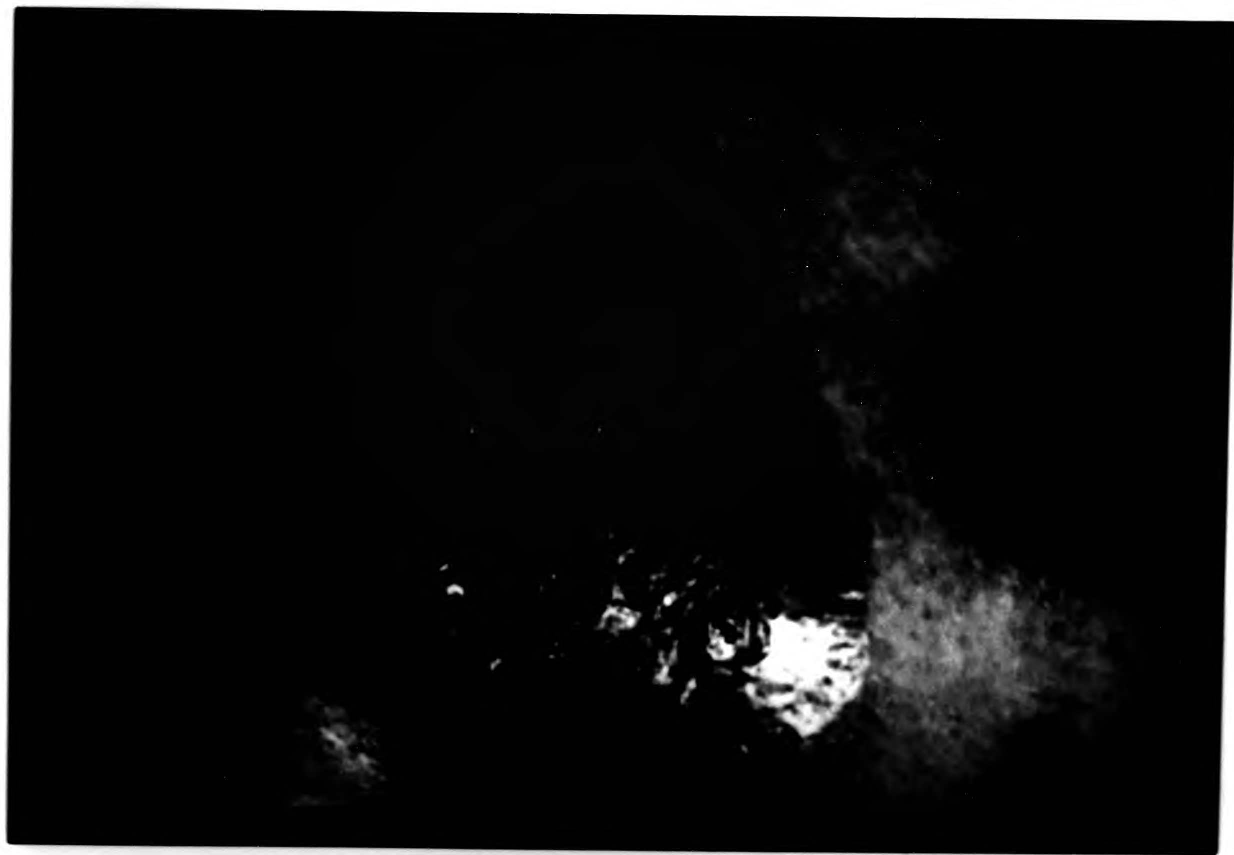
It was often difficult to distinguish between colonisation and infection in these patients. Among the clinical signs associated with infection by either organism were: a red flare surrounding the raw area (Fig. 34), an elevated temperature (although many burnt patients are apparently pyrexial in the absence of infection), positive blood cultures, septic arthritis and destruction of a skin graft (Fig. 34). Clinical advice and photographs were provided by Mr J.A.Clarke, consultant surgeon to the unit. When these streptococcal infections occur it is not possible to ascertain by clinical criteria alone which of the agents is responsible (Pers.comm.M.Weinbren).

The area of the body surface affected by burns ranged from 5% to 90% in these patients. Infection with group A or group G streptococci was thought to contribute to the cause of death of 18 of the 759 patients (2.5%) admitted to the unit (Table 54). These were either patients with excessively severe burns who died soon after admission or who subsequently developed septicaemia. The ages of the patients ranged from 18 to 89 years but one half were more than 60 years of age. Of the 18 fatal cases group G streptococci were isolated from 15, group A streptococci from four and both were isolated from one patient.

Group G streptococci were isolated throughout the study with the

Fig.34 Invasiveness of group G streptococci in burns and skin grafts





These photographs were kindly provided by the Burns Unit QMH, London.

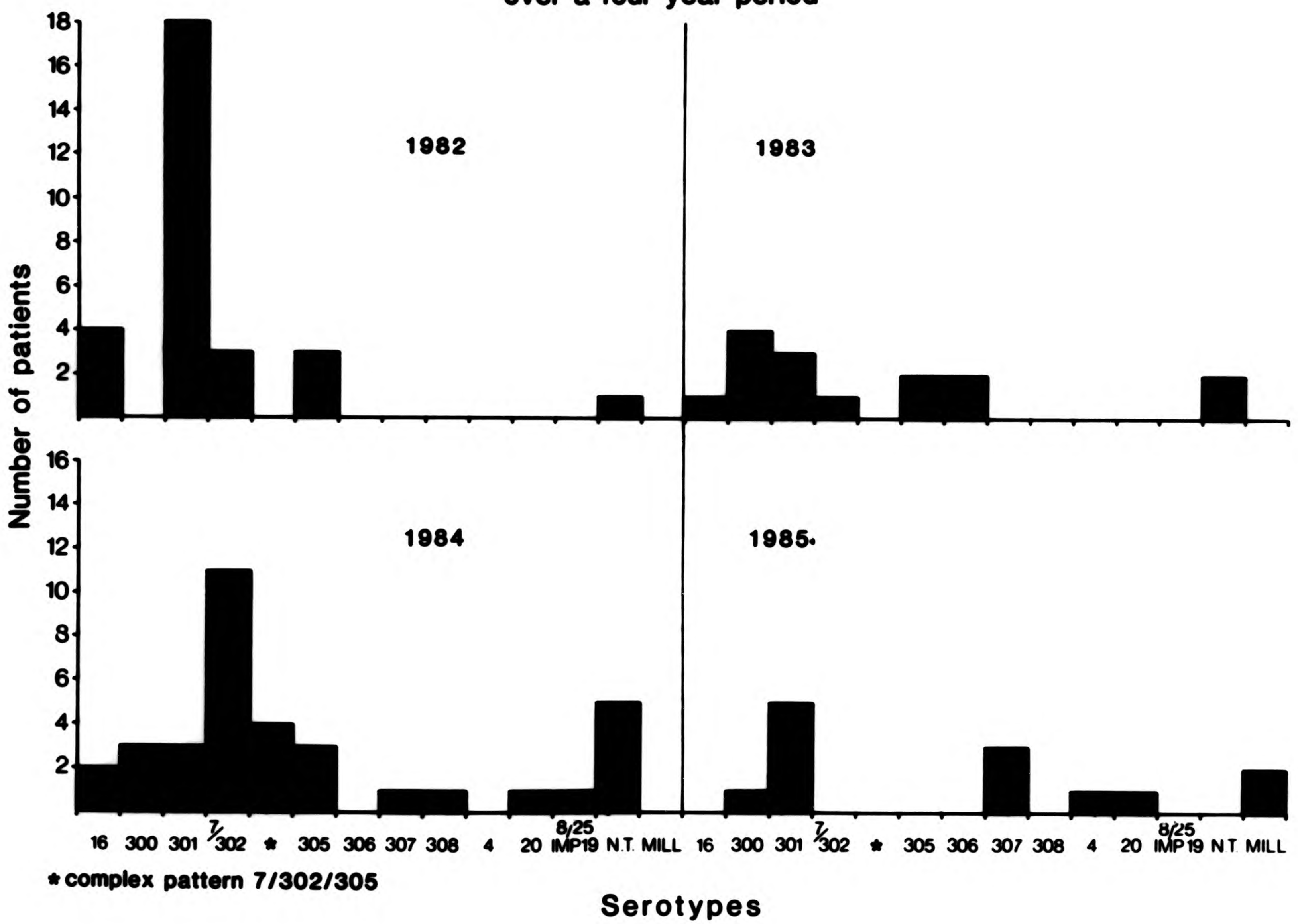
Table 54

Incidence of mortality in a burns unit over a four year period

Patient's age	Sex	% Burn	*Days after admission	Raw area group A	infections group G	T type
62	F	20	47	-	+	301
41	F	†	†	+	-	‡NR
26	M	†	2	+	-	MT63
49	F	60	12	-	+	NR
70	F	35	3	-	+	301
31	F	†	21	+	+	NR
70	M	20	†	-	+	N.T.
87	F	30	0	-	+	301
89	F	†	†	+	-	MT9
74	M	†	61	-	+	NR
68	M	25	34	-	+	301
18	F	27	†	-	+	NR
74	F	5	13	-	+	301
37	M	90	2	-	+	NR
54	M	†	29	-	+	NR
44	F	70	6	-	+	8/25/IMP19
31	F	60	2	-	+	N.T.
26	M	75	2	-	+	301

*: Death after admission (in days)
†: Information not given
‡NR: Strain not sent for serotyping

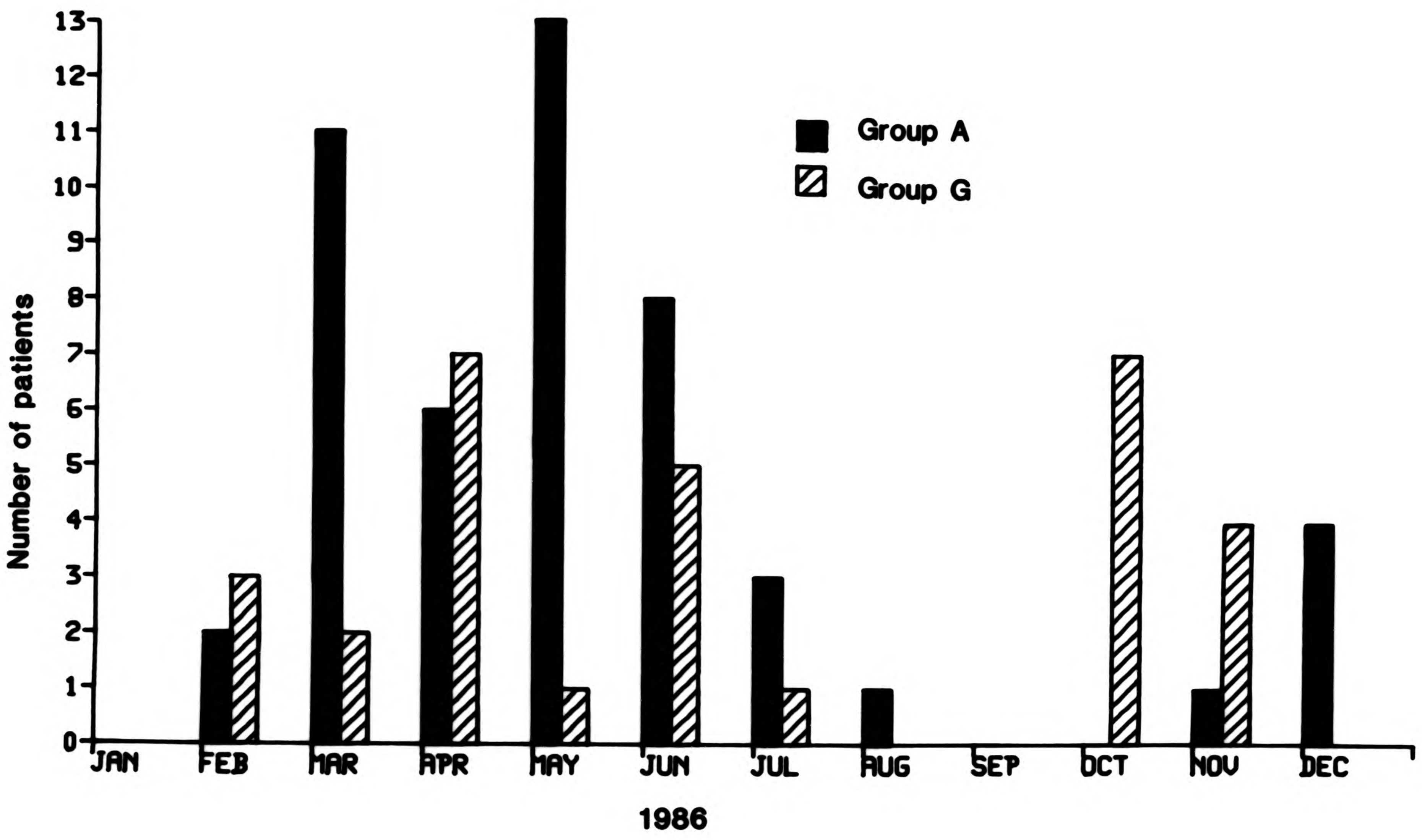
Fig. 35 Serotype distribution of Lancefield group G streptococci in a London Burns Unit over a four year period



exception of swabs taken during four months (Fig. 33). The typability rate for group G streptococci was 93% for strains referred to this laboratory. Certain serotypes predominated in particular years (Fig. 35). In 1982, for instance, serotype 301 was the most numerous single type and it was the failure of skin grafts associated with infection with this serotype that drew attention to the problem. The deaths associated with infection with this serotype that are listed in Table 54 occurred during the years 1982 and 1983. Strains with an unusual pattern of agglutination were isolated. These strains which were agglutinated by the sera numbered 7, 302 and 305 were the only representatives with this pattern handled and were isolated from raw areas of four patients who were in the unit at the same time.

There is no apparent decrease in the number of patients infected with either group A or group G streptococci following the transfer of the unit to purpose-built accomodation. The histogram forming figure 36 shows the number of patients per month during the year 1986 that were infected with strains of one or other Lancefield group. In April of that year serotype 7/302 predominated being isolated from six of the seven patients from whom group G streptococci were cultured. In June all five patients colonised, or infected, with this organism yielded representatives of serotype 300. The same serotype was recovered from two patients in October and serotype 305 from the other four patients. During the first half of 1986 there was a concurrent outbreak caused by strains of Lancefield group A, serotype 73. This serotype was recovered from

Fig. 36 Continuing infections in a London Burns Unit (1986)



all patients infected with group A streptococci, the environment and from the nose and throat swabs taken from members of the staff of the unit.

Strains from the burns and plastic surgery unit in a hospital in Essex

A total of 43 strains of Lancefield group G and 36 of group C were studied from a hospital in Essex. Isolates from both patients and members of staff were available for study (Table 55). These strains were isolated over a period of nine months. Cross-infection was suspected when large numbers of group C or group G streptococci were isolated from different patients at any one particular time within the combined unit. Serotyping results were consistent with this view. For instance, Lancefield group G serotype 21 strains were isolated contemporaneously from the wounds of three patients and two members of staff in the burns ward, and from five patients in the plastic surgery ward. Later, serotype 308 was found in the wounds of three patients with burns and four patients in the plastic surgery unit. There were in addition two outbreaks of infection caused by strains of group C, serotype 21 in the unit. The first involved nine patients. The second incident occurred several weeks later with four further cases of sepsis, including one in which there was failure of a skin graft. Throat cultures were taken from the staff in attendance during the second incident and three carried representatives of serotype 21.

Outbreaks of skin sepsis in hospital wards

The most numerous clusters of strains of Lancefield group G

Table 55

Serotype distribution of group C and group G streptococci from a collection of strains in a burns and plastic surgery unit in Essex

	Serotypes																		
	Lancefield group C						Lancefield group G												
	20	21	203	305	306	*N.T.	16	20	21	300	301	302	303	304	306	307	308	*N.T.	+Mill.
Regional burns unit	1	13	3	2	1	0	1	.	3	.	1	5	.	.	1	.	3	0	1
Regional burns unit staff	.	3	5	.	.	1	1	.	2	.	1	1	1	.	.	.	.	0	.
Plastic surgery unit	.	1	2	4	.	0	3	.	5	1	2	.	1	3	.	1	4	2	.

* Not typable
+ S.milleri

received from hospitals had originated in bacteriological studies of skin sepsis. The origin and serotype distribution of the 102 strains received from 19 hospitals are summarised in Table 56. In all patients clinical signs of infection were present and this had developed at sites of previous damage to the skin. There was no suggestion in any instance that the streptococci had been isolated in pure culture. The non-typable strains apart (17 cultures) the most frequently represented types were 301 (17 cultures), 7/302 (14 cultures) and 303 (11 cultures). Of these the strains of serotypes 7/302 and 303 were heavily represented among those received from Hospital S.

Outbreaks of infection in a London ulcer clinic

A total of 30 strains of group G streptococci were isolated from ambulatory patients attending an ulcer clinic. These were patients with infected varicose vein ulcers. The patients were received into the clinic in groups, dressings were removed and the infected wounds were left exposed whilst the patient was waiting for examination. Over a period of 12 months a total of 15 representatives of serotype 305 were isolated. This organism was isolated from approximately three-fifths of all patients attending the clinic. The organism was isolated also from the floor of the clinic particularly beside the examination couch and from the plastic aprons worn by the nursing staff in attendance. The method of transmission was not investigated but aerial transmission of skin flakes is one explanation. Serotype 7/302 was isolated from the skin of six patients attending the clinic at the same

Table 56

Outbreaks of skin sepsis in hospitals due to Lancefield group G streptococci

Apparent outbreak	Source	No. of isolates (Total of 102)	T type
Hospital A			
Plastic surgery unit	Skin grafts	2	201
		2	N.T.
Hospital B			
Ward: patients	Sore hip	1	All
	Pin site	1	301
	Blisters	1	
Hospital C			
Ward: patients	Sores	3	All
environment	Floor, chairs, scales	6	7/25
Hospital D			
Plastic surgery unit	Skin grafts	4	301
Hospital E			
Ward: patients	Wound	1	303
	Skin grafts	2	20
	Foot ulcer	1	N.T.
Hospital F			
Plastic surgery unit	Skin grafts	2	301
		2	N.T.
Hospital G			
Ward: patients	Impetigo	1	N.T.
	Leg ulcer	1	N.T.
Hospital H			
Ward: patients	Skin sores	5	301
Hospital I			
Geriatric intensive care unit	Wounds (females)	3	N.T.
	" (male)	1	301
Hospital J			
Ward: patients	Skin sores	1	N.T.
		1	7/302
Hospital K			
Dermatology unit	Skin lesions	6	308
Hospital L			
Ward: patients	Leg ulcers	2	305

Hospital M			
Psychogeriatric ward	Pressure sores	1	21
		1	16
		1	301
		1	N.T.
Hospital N			
Ward	Elbow lesions	1	All
	Leg sores	4	304
	Heel swabs	2	
Hospital O			
Plastic surgery and burns units	Pressure sore	1	All
	Wound	1	7/302
	Burn	1	
	Sputum	1	
Hospital P			
Ward	Tracheostomy swab	1	16
	Skin graft	1	N.T.
	Wound	1	7/302
Hospital Q			
Ward	Leg ulcers	4	305
		1	308
Hospital R			
Medical unit	Skin lesions	1	305
	" "	2	7/302
	" "	1	301
	Sputum	1	S.milleri
Hospital S			
Wards 1 and 2	Infected grazes	4	7/302
	Skin sores	3	303
		1	N.T.
Ward 3	Leg ulcer	1	20
	"	1	N.T.
	Sore on foot	1	7/307
Ward 4	Skin sores	3	303
	in	1	21
	Psychiatric	1	N.T.
	ward	2	7/302
Ward 5	Leg ulcers	2	7
Wards 6 and 7	Skin lesions	4	303
	Sore on foot	1	N.T.

time. There were two isolations of type 303, one of type 21 and six strains that were not typable with the set of antisera.

Group G streptococci in the West Indies

Between January 1976 and July 1980 of the 1231 strains of haemolytic streptococci referred by the Trinidad and Tobago Public Health Laboratory, Port of Spain, Trinidad to this laboratory 63 belonged to Lancefield group G. Of these isolates 52 were from patients with acute glomerulonephritis or their siblings and the other 11 isolates came from one patient with rheumatic fever and ten siblings (Reid et al. 1985). These strains were not available for the study but 15 cultures of Lancefield group G and five of group C that had been referred later were. The cultures of group C (*S.equisimilis*) were all isolated from different members of the same family and all belonged to serotype 203. Two cultures of group G were known to be from throat and skin swabs taken from a patient with rheumatic fever and both belonged to serotype 306. The other 13 cultures of group G, like the representatives of serotype 203 of group C, were isolated during surveys and three carried the T2 antigen, three were agglutinated by the 7/302 sera, two belonged to serotype 308 and five were not typable.

Group G streptococcal infections in maternity units

A total of 70 strains were isolated from infections in maternity units that occurred in seven hospitals (Table 57). A large outbreak of puerperal sepsis involving 12 patients occurred over a period of two weeks at hospital F. Serotype 7/302 was isolated

Table 57

Group G streptococcal infections in Maternity Units

Apparent outbreak	Source	No. of isolates (total 70)	T type
Hospital A			
Mother (a)	Offensive lochia	1	All
Baby (a)	Nose	1	7/302
Mother (b)	Offensive lochia	1	
Hospital B			
Mother	HVS	1	All
Baby	Gastric aspirate, ear, rectal swab	3	20
Hospital C			
Babies in unit at same time	Cord (baby 1)	1	All
	Umbilicus (baby 2)	1	301
Hospital D			
	Post natal and	3	301
	Perineal infections	5	7/302
		1	7/25
		1	28
Hospital E			
	Patients (post natal)	9	308
	"	2	7/302
	Staff member (throat)	1	308
	Sister on unit	1	N.T.
	Midwife (nose)	1	N.T.
Hospital F			
	Patients (HVS and	12	7/302
	MSU)	1	21
	Wounds	2	300
	HVS	1	20
Hospital G			
Patients	Wounds	2	20
	Wounds	3	21,28,301
	Perineum	10	7/302
Environmental	Bath water	2	7/302
	Bath, toilet seats	4	7/302

from all patients with the exception of one isolate which carried the serotype 21. The outbreak was cleared without the source being found. A few months later, there were three further cases but different serotypes were isolated from a caesarian wound and high vaginal swabs. Representatives of serotype 7/302 were isolated also from outbreaks in hospitals D and G. In the latter hospital representatives of this type were isolated from perineal infections in ten mothers and also from environmental swabs taken in the bathroom they shared. Five additional mothers developed infections with representatives of types 20, 21, 28 and 301. In hospital D cultures representing four different serotypes were isolated from vaginal and perineal swabs taken from ten patients. The serotype 308 was established for strains isolated from an outbreak of infected episiotomy wounds in hospital E. A culture of the same type was isolated from the throat of one member of staff and two members of the nursing staff carried non-typable strains. Cultures of the serotype 7/302 were isolated from two further patients in the ward.

Colonisation of babies with strains carried by their mothers were noted in hospitals A and B and skin infections of two babies who were in the neonatal unit contemporaneously occurred in hospital C.

<u>Asymptomatic carriage in institutions</u>

The prevalence of pyogenic streptococci of the Lancefield groups A, C and G in the nose and throat was tested in Home Office establishments. The carriage rates for group G streptococci

approached 10% (Table 58). In the first establishment ten of the 17 strains belonged to serotype 300 and in the second, nine of the 20 strains were identified as type 308. In each institution a total of seven different serotypes were recovered (Table 59). It would seem from these findings that Lancefield group G streptococci could be passed from person to person without necessarily causing disease. Asymptomatic pharyngeal carriage of group G streptococci occurs in up to 23% of humans (Hill et al. 1969).

Foodborne outbreaks of group G streptococcal pharyngitis

Foodborne outbreaks of group G streptococcal sore throat are rare. Strains were obtained from what were thought to be two such outbreaks - one in the U.S.A., the other in Israel. The first occurred in Florida, amongst persons who had attended a convention at a hotel (Stryker et al. 1982). Seventy two (31%) of the 231 participants who were interviewed became ill, and group G streptococci were isolated from ten of 16 persons with pharyngitis and one of 41 of persons without. Ten strains were available for serotyping and all belonged to type 301. The evidence obtained from a case controlled study favoured a common source outbreak (Stryker et al. 1982) with a median incubation period of two days. Chicken salad was eaten by all those who developed sore throat and the attack rate among those who ate the salad was 53% (57 of 108). Group G streptococci of serotype 301 were isolated from the cook who had prepared the salad but she did not become ill until after she had prepared the meal. The second outbreak occurred in an army camp in Israel. Organisms referred for serotyping were

Table 58

Asymptomatic carriage of group G streptococci in two residential Institutions

	No of throat swabs	Streptococcal isolates A	C	G
Institution A	182	5	14	17
Institution B	220	19	7	20

Table 59

Serotype distribution of Lancefield group G streptococci in asymptomatic carriers

	Serotypes 16	20	300	301	7/302	304	306	307	308	N.T
Institution A	1	1	10	1	1	1	0	0	2	0
Institution B	0	0	2	2	4	0	1	1	9	1

recovered from four soldiers with sore throat, two kitchen workers and from meat balls all the affected had eaten. None of group G streptococci from this outbreak could be serotyped with the set of antisera available.

The isolation of Lancefield group C and group G streptococci from clinical specimens in two hospitals

The type distribution among hospital strains of Lancefield group C and group G streptococci was studied with the help of two clinical laboratories one in Scotland (RH), the other in London (NPH). There was no interchange of patients between the two hospitals. What were thought to be random isolates of group C and group G from a variety of infections were referred for serotyping. All were of human origin. Multiple strains of a single serotype isolated on different occasions from a single patient were accepted as one isolate. Groupable strains which exhibited 'minute' colonies on blood agar and which could not be serotyped were identified biochemically with the aid the API 20 STREP system. They were, without exception strains of S.milleri.

A total number of 326 independent isolates were provided. From hospital NPH (London) there were 34 strains of group C streptococci and 150 of Lancefield group G. At hospital RH (Scotland) there were 65 isolates of group C streptococci and 77 of group G. The typability rates from both hospitals were similar. For the Lancefield group C streptococci, 88% of isolates from the south were typable and 89% of isolates from the north could be serotyped.

Whereas, 78% of Lancefield group G streptococci could be serotyped from the south, 83% from the north. There was a wide range of typing patterns for both groups, with strains from the north being rather more evenly distributed (Figs. 37 and 38). The largest groups of isolates examined were from the throat, ulcers, wounds, skin, HVS and the ear (Table 60). The overall serotype distributions (%) for both hospitals is shown in Fig. 37.

Group C streptococci

The largest number of group C streptococci examined were from the throat and belonged predominantly to the serotype 21. Serotypes 21, 203 and 305 were the most common types found in the throat from patients in the north. Similarly, type 21 was equally as common in the south, type 301 was also frequently isolated. Of isolates from the north 35% were from the throat and 23% belonged to the species S.milleri.

Group G streptococci

The group G streptococci from either hospital could be subdivided into at least 15 different serotypes. S.milleri represented 3 of the 77 (4%) strains from the north - one from the throat and the other two from sputum. The serotype distribution pattern varied for each hospital. The most common serotypes for hospital NPH appeared to be types 7/302, 301, 308 and 16. For hospital RH, serotypes 7/302, 308, 300 and 7/307 appeared to predominate (Fig. 38). The largest groups of isolates were from the throat, ulcers, skin, HVS, wounds and the ear. Table 60 lists the type distribution of group G streptococci from both hospitals by site of infection.

Fig. 37 Serotype distribution of Lancefield group C and group G streptococci at two hospitals in the U.K.

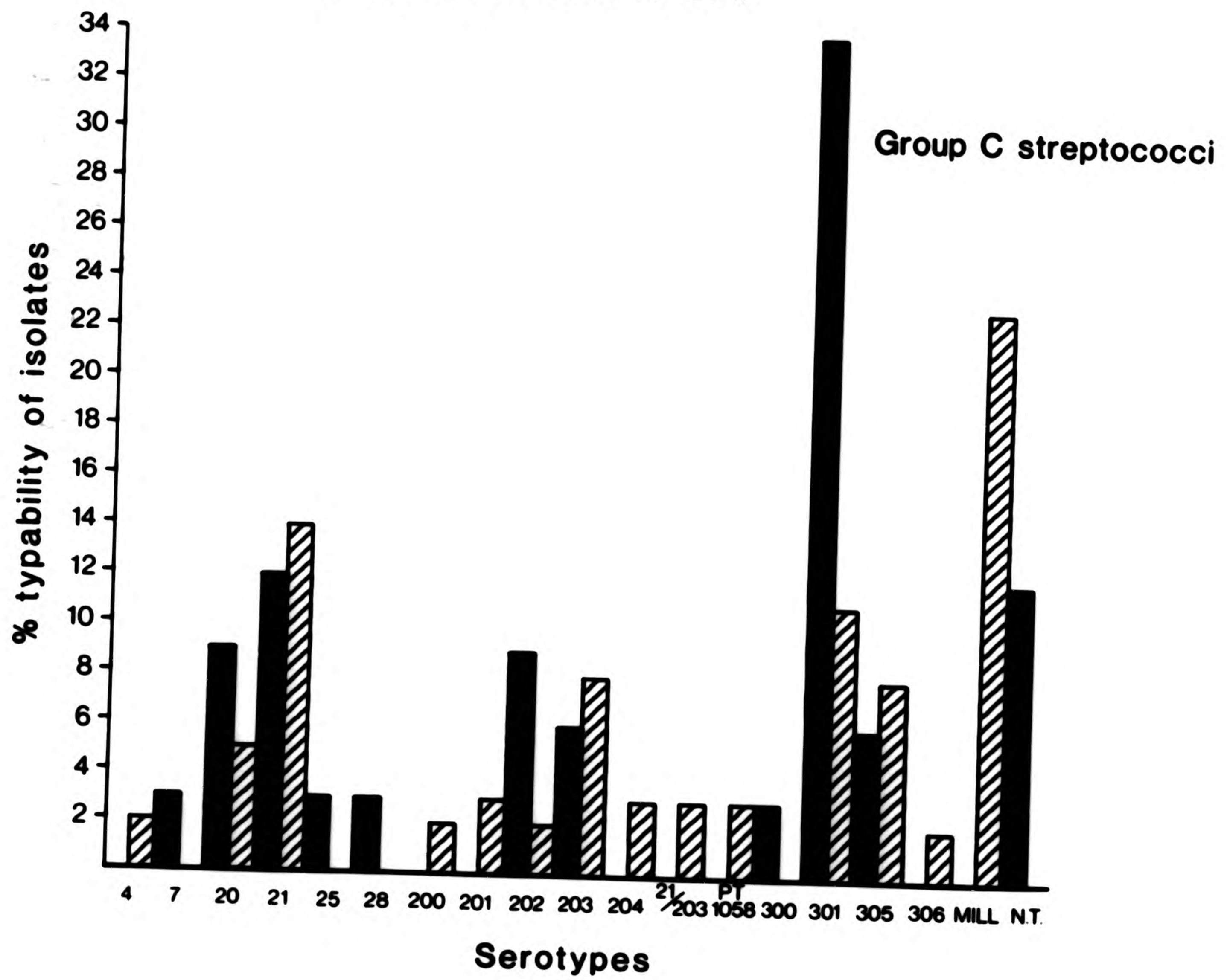


Fig. 38

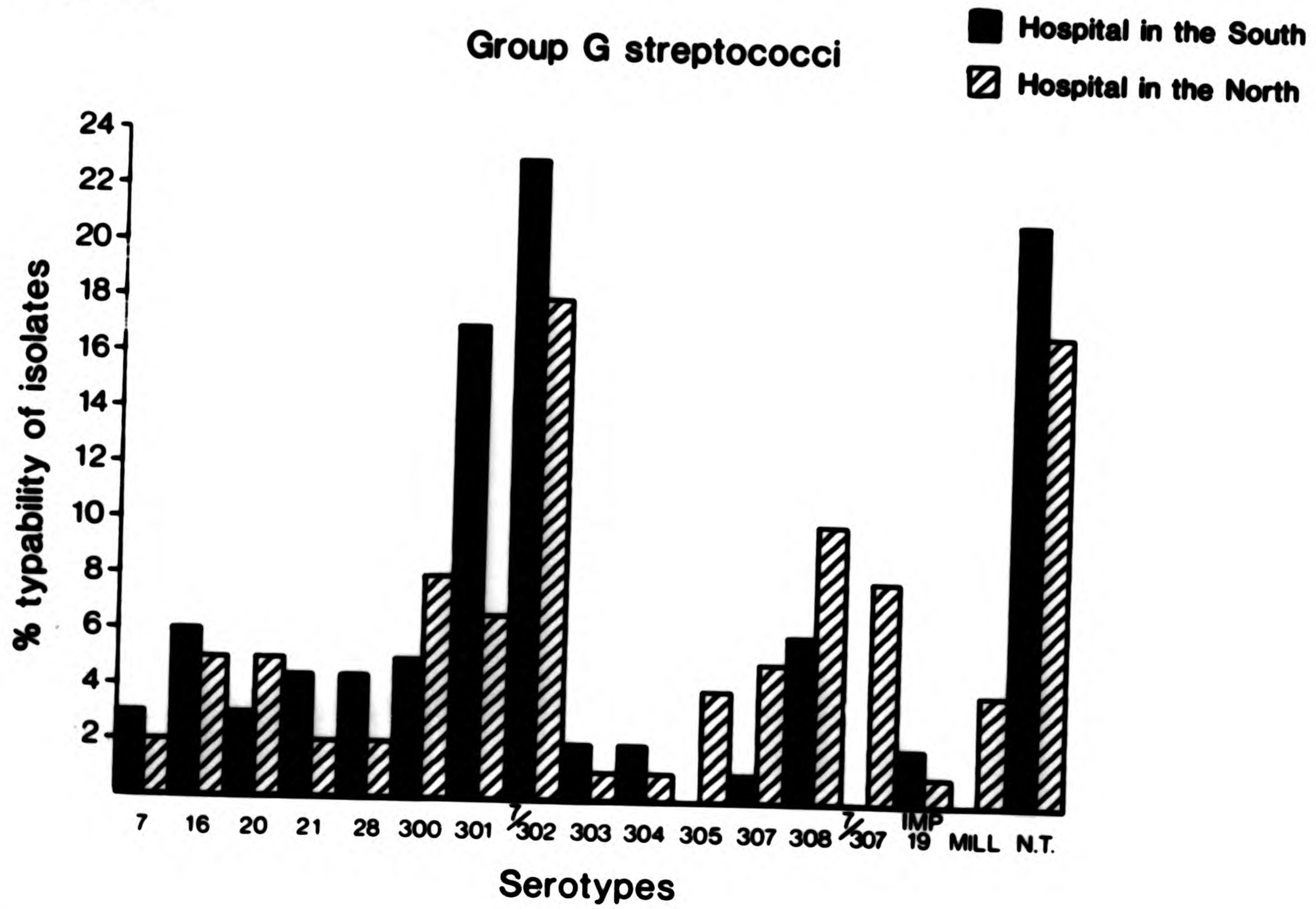


Table 60

Distribution of serotypes of Lancefield group G streptococci isolated from common sites in two independent hospitals - one in Scotland (RH) and the other in London (NPH)

Serotype	Source											
	Ear		Urinary tract		Skin		Throat		Ulcers		Wounds	
	S	N	S	N	S	N	S	N	S	N	S	N
16	.	.	1	2	3	.	2	1	.	.	.	.
300	1	.	1	1	.	2	2	2	.	.	4	.
301	.	.	2	.	6	1	3	2	6	.	2	.
7/302	2	2	6	3	9	1	1	2	3	.	2	3
303	.	.	.	.	2	.	.	.	1	1	.	.
304	.	1	.	.	1	.	1	1	.	.	.	.
305	.	.	.	1	.	.	.	.	.	.	.	1
307	1	1	.	2	.	.	.	1	.	.	.	.
7/307	.	1	.	1	.	.	.	.	.	2	.	2
308	.	1	2	2	2	1	2	.	.	.	1	1
7	.	.	1	1	.	.	.	.	1	.	.	1
20	.	1	1	1	.	1	.	.	.	.	1	.
21	.	1	2	.	.	.	.	.	.	.	1	.
28	.	.	.	.	.	.	.	.	6	.	.	.
8/25/IMP19	.	.	.	.	.	.	.	1	1	.	.	.
S.milleri	.	.	.	.	.	.	.	1	.	.	.	.
Not typable	3	1	1	3	1	1	6	5	8	2	7	.

Strains which reacted with typing sera usually associated with Lancefield group A or C streptococci were not common, with one exception. These were isolated in London from leg ulcers and carried the T-antigen 28 (which is a Lancefield group A serotype). Also, it was evident, with strains from the two sources that the complex pattern 7/302 was common amongst clinical isolates of Lancefield group G.

Summary

A collection of 1777 strains belonging to one or other of Lancefield group C or group G was examined. These cultures had been isolated either sporadically from patients with serious disease or as apparent clusters from various institutional outbreaks. Strains from four prospective studies were examined. Two were from district hospitals, the others were from specialist burns units. T-protein antigens were sought as a means of subdividing these strains. The occurrence of these antigens in strains of both Lancefield groups was confirmed. Of the human isolates examined, 87% of group C streptococci and 82% of group G could be serotyped by this method. There were several instances of cross-over between T antigens belonging to the two Lancefield groups with strains carrying T antigens usually associated with the other group. This was also seen with some strains of Lancefield groups C or G which carried the T antigens, 2, 4, 28 or 8/25/IMP 19 normally found in strains of group A.

Antibiotic sensitivities of random isolates of Lancefield group C and group G streptococci

A limited study of antibiotic sensitivities was made on a total of 404 strains of Lancefield groups C and G streptococci of human origin from a variety of sites. They were screened for their antibiotic sensitivity patterns against five antibiotics. A disc sensitivity method was used with discs containing penicillin (1 unit), chloramphenicol (10 µg), tetracycline (5 µg), erythromycin (0.5 and 10 µg) and oxacillin (1 µg).

All strains were judged sensitive to penicillin, oxacillin and chloramphenicol. Tetracycline resistance, represented by the absence of a zone of inhibition, was found in 55% of strains. Without exception strains carrying the T-type complex pattern 7/302 were resistant to tetracycline. Resistance to erythromycin was found in 6% of strains. These were from leg ulcers (3%) and skin infections (2%). The remaining 1% included isolates from the throat, blood and urine. Overall, 2% of the group C streptococci examined were resistant to erythromycin and 4% of group G were also erythromycin resistant. The 3% of strains from leg ulcers which showed erythromycin resistance belonged to serotypes 28 and 303 for group G and types 301 and PT1058 for the Lancefield group C. The remaining 3% of erythromycin resistant strains belonged to Lancefield group G and carried the serotypes 20, 300 and 301. Three isolates could not be typed with the set of antisera available.

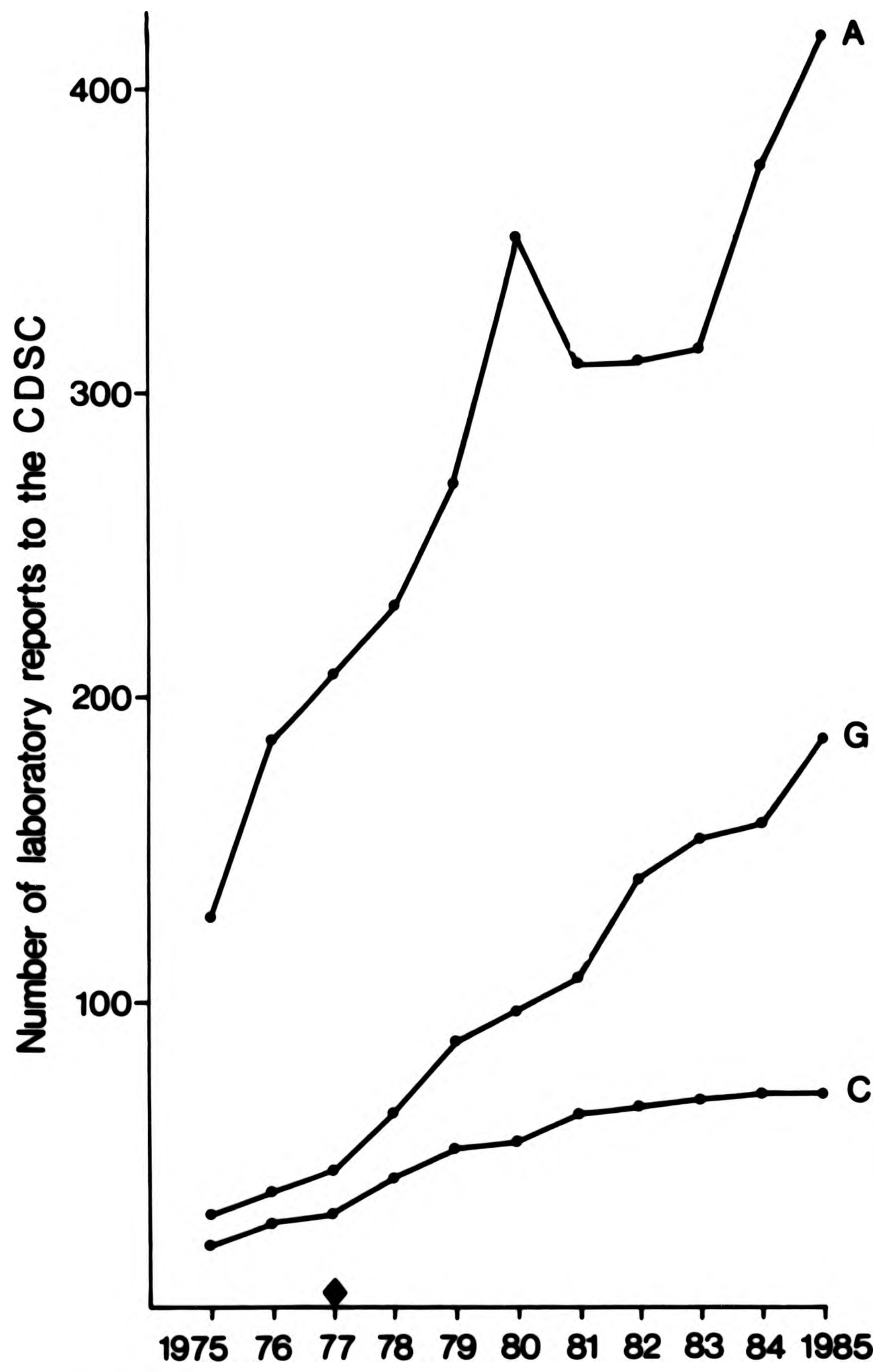
4. DISCUSSION

4. DISCUSSION

The pyogenic streptococci of Lancefield group C and group G were recognised as a cause of epizootics in closed animal populations (Tillman et al. 1982) long before they were considered as agents of infectious disease in humans. Prior to 1980 there were few descriptions of cases of endocarditis, septic arthritis, sore throat, cellulitis and septicaemia in man. The studies of Lancefield and Hare (1935) of infections in parturient women provided a serological method for distinguishing these strains from S.pyogenes. Their work clearly established that representatives of Lancefield group A (Streptococcus pyogenes) were the cause of the life-threatening infections in these women and it seems possible that this led microbiologists to underestimate the harm that streptococci of group C and group G could do.

Since 1980 there has been an increasing awareness of these organisms as pathogens. On the one hand this had led to an increase in the number of published case reports and on the other to more notifications of serious infections to the Communicable Disease Surveillance Centre (CDSC) from laboratories in England and Wales. The graph (Fig. 39) illustrates this trend for increased reporting and the isolates were predominantly from blood cultures. Perhaps not unexpectedly reports of Lancefield group A strains (S.pyogenes) predominate (Table 61). The number of reports of infections caused by group C streptococci has remained relatively stable since 1981 whereas there has been an increase in the number

Fig. 39 Streptococci: Laboratory reports to the CDSC



◆ The introduction of rapid streptococcal grouping kits.

Table 61

Laboratory reports of pyogenic streptococci of Lancefield groups A, C and G to the C.D.S.C. 1975 - 1986

% no. of infections caused by pyogenic streptococci reported

Group	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986
A	72	74	73	68	66	70	64	60	57	56	62	60
C	11	11	11	12	13	11	13	13	15	12	10	
G	17	15	16	19	21	19	23	27	28	26	28	*40

* Individual figures for group C and group G not yet available from C.D.S.C.

Public Health Laboratory Service, Communicable Disease Surveillance Centre, (Unpublished data).

of reports of infections caused by either group A or group G. This trend is probably due in part to the availability of commercial kits for the identification of Lancefield group antigens during this decade and also to an increase in the total number of reports. As an example of the latter, the number of reports of bacteraemias caused by any organism rose from 11,115 in 1980 to 20,559 in 1985 (CDSC, unpublished data). It would seem that these streptococci of Lancefield groups C and G are only now being recognised as significant human pathogens and the main aims of this study were to provide methods that could be used in epidemiological investigations of apparent outbreaks, to identify properties that are associated with strains of particular virulence and to find new tests that would give clearer descriptions of the various species within these two Lancefield groups.

Despite the recent reports of isolation from disease there is a need for a well-defined typing scheme similar to that established for S.pyogenes (Maxted 1978). Such a scheme could have been applied, profitably, to what appear to have been common source outbreaks in veterinary (Tillman et al. 1982) and human medicine (Hill et al. 1969, McCue 1982, Stryker et al. 1982). When a typing scheme is used during the investigation of an apparent outbreak the assumption is made that isolates of the same type are usually related epidemiologically (Parker et al. 1976). Useful and valid schemes have been based on cell wall antigens among, for example, the Enterobacteriaceae and the group A streptococci. It would seem that structural components of the bacterial cell are

more likely to be stable markers than for example, sensitivity to bacteriophages or bacteriocins. For the group A streptococci the cell wall associated T- and M-protein antigens provide the basis for the established serotyping methods. The T-protein antigens are not known to be related to virulence but the identification of these antigens has been useful, for instance, in the study of strains isolated from cases of acute post-streptococcal glomerulonephritis (Parker et al. 1968). It was several years before methods were developed that permitted the demonstration of M antigens in these strains (Pranitis et al. 1973). The M-protein antigens are clinically significant because immunity to S.pyogenes is at least in part type specific and is governed by these antigens (Maxted 1978). A typing scheme based on virulence factors has an inherent attraction because of its direct clinical relevance. In practice, a combined approach based on the detection of both T and M antigens has been particularly successful with group A streptococci (Williams and Maxted 1955) and a similar approach for the pyogenic human streptococci of group C and group G would be an obvious beginning.

Griffith (1934) based his typing scheme for S.pyogenes on what came to be known as T-protein antigens. His work pre-dated the introduction of the Lancefield grouping procedure and his serotypes 7, 16, 20 and 21 were later recognised as occurring in strains of group C and group G only. These antigens were not sought in reference laboratories but provided a starting point for this study.

To test the possibility that group C and group G streptococci did indeed possess T-protein antigens, a collection of 1777 strains was made from a variety of sources. Some were single non-related isolates and others were collections of strains from possible outbreaks. A total of 20 different T-protein antigens have so far been recognised using specific polyclonal rabbit antisera. When the serotyping scheme was applied to outbreak strains it was possible to show that there was good evidence of spread of a single strain within each outbreak. In some instances it was possible to suggest the origin of the infecting strain.

4.1. INFECTIONS CAUSED BY LANCEFIELD GROUP C STREPTOCOCCI IN MAN

Even though the carriage rate of group C streptococci in normal individuals was stated to be as high as 3% (Hare 1940), disease caused by these organisms in humans is still thought to be relatively uncommon (Stamm and Cobbs 1980). In this study the ratio of group C to group G streptococci causing infection was 32% to 68% respectively. In contrast Kirby and Rantz (1943) found that group C streptococci were second only to group A as a cause of human infection.

The serotyping scheme developed allowed the division of group C strains from random isolates into 16 different categories with a typability rate of 87%. A total of 18% carried T antigens also common to strains of Lancefield group G and 1% carried T antigens

first described in strains of group A. These overlaps may reflect antigenic similarities even though the antisera developed for the scheme did not react with any of the group A serotypes.

The largest groups examined were from the throat and from skin ulcers. As group C streptococci can exist asymptomatically in the upper respiratory tract (Rantz 1941), it was not surprising to find that 39% of isolates were from cases of pharyngitis or tonsillitis. Approximately 6% of the group C streptococci received were from serious systemic disease. The majority of these were from blood cultures from cases of septicaemia or endocarditis. Duma et al. (1969) quoted 0.6% of all streptococcal isolates from the blood as group C. The C.D.S.C. figures for the percentage number of serious infections caused by group C streptococci are illustrated in Table 61. The table shows that during the period 1975-1986, 10-11% of pyogenic streptococci causing serious disease, belonged to Lancefield group C (the majority of which were from the blood). Compared to the streptococci of Lancefield groups A and G, serious systemic disease was not very common. Infections included mainly bacterial endocarditis, cellulitis, septicaemia, pneumonia, nephritis and meningitis. From the clinical data received it would appear that these infections showed great similarities to those caused by the group A streptococci. The reported cases of group C streptococcal endocarditis were said to follow an acute destructive course requiring strict monitoring for signs and symptoms of early cardiac decompensation (Feinberg and Shabino 1985). Empyema is stated to occur in all cases of pneumonia due

to group C streptococci (Stamm and Cobbs 1980), as apparently often happens in group A streptococcal pneumonia (Mohr et al. 1979). It seems that in the 15 cases of cellulitis (examined in this study) the clinical manifestations of the infections from which group C streptococci were isolated were indistinguishable from cellulitis caused by other pyogenic streptococci. AGN and meningitis were rare in this study and generally from the data reported in the literature. Rheumatic fever after group C infections has not been reported even though AGN can occur (Basset 1972, Mihalcu et al. 1982). In this study there was one throat isolate of group C from a patient who had acute rheumatic fever and a sore throat. The strain was non-typable, perhaps a new serotype due to the rarity of the infection. Overall there did not appear to be any particular association of serotype with a systemic or infectious disease.

Outbreaks of infection due to group C streptococci are unusual. Strains were received from outbreaks in institutions, two schools, one army camp and three hospital wards. The largest single outbreak of sore throat studied using this scheme involved 146 members of staff from one hospital. Female members of the nursing staff who had eaten salads containing cold hard boiled eggs were infected. Foodborne outbreaks due to group C (S.equisimilis) are apparently rare because they have not been documented in the literature. The serotype responsible for this outbreak (type 204) is uncommon within the community as judged by the study of random isolates of group C (Table 47).

Group C streptococci have not been described as a cause of outbreaks of puerperal sepsis which is usually attributed to the Lancefield group A streptococci. However, there was one very recent outbreak of puerperal sepsis during this study involving 50 women. Representatives of serotype 204 were isolated from the genital tract of each of the 50 women involved and also from environmental swabs taken in the bathrooms used by these patients. Isolates were also obtained from two members of the nursing staff who developed sore throats. The outbreak is presently still under investigation (Pers.comm.E.L.Teare). The only other known outbreak caused by this type was in Romania where sore throat infections were followed in some instances by cases of nephritis (Mihalcu et al. 1982). It may of course be fortuitous but all isolations of serotype 204 were from outbreaks of disease. Representatives of strains from pharyngitis cases, tonsillitis cases, nephritis cases and carriers from the Romania outbreak were subjected to three typing schemes in a collaborative study. Thus, the T-typing scheme was tested against a phage typing scheme for S.equisimilis and the DNA 'fingerprinting' of the organisms involved. The schemes produced similar results which indicated that strains of serotype 204 were identical, with the sole exception of the case of pharyngitis caused by a strain of serotype 21. However, it seems that the identification of the T protein is an easier method to establish, reproduce and apply than the other two schemes. In addition, the typability rate for group C by T-protein typing (87%) is far greater than the rate quoted for the lysotyping scheme (71%) (Mihalcu et al. 1982). From all these

results it was not possible to subdivide further the serotypes by phage typing and further the additional procedures did not provide any indication or information on the invasive properties of the organisms.

A complementary scheme to T serotyping which could provide information not only on the epidemiology but also the identification of possible virulence markers for example, M proteins, would be extremely valuable. An established scheme based on the identification of M proteins could also improve the present discriminatory power of the system. The discriminatory power of a typing scheme is said to be inversely proportional to the frequency of the most common type (Parker et al. 1976). Ideally the 'perfect' typing scheme should be able to divide the species into small, evenly sized groups. However, few serological methods can achieve this. For the T-typing of group C streptococci the most common serotypes were types 21 and 301 which comprised 20% and 23% of the total distribution respectively. Thus 43% of isolates belonged to one or other of two serotypes. Greater discrimination could be achieved by the introduction of a complementary typing scheme. As it stands however, the application of T-typing as an epidemiological marker has shown that streptococci of Lancefield group C (S.equisimilis) of a single serotype can be isolated from outbreaks of infection.

Human infections caused by other species of group C streptococci are uncommon. There have been however recent reports of outbreaks

due to S.zooepidemicus. All of these outbreaks involved either the consumption of unpasteurised dairy products or some association with animals. It would seem that they should be regarded as zoonoses. In some patients, infections with S.zooepidemicus were overwhelming (Ghoneim and Cooke 1980, Barnham et al. 1983). In view of their severity these infections have been described as the most notable milk-borne disease of the last few years in the U.K. (Sharp et al. 1985). Some of the patients who developed pharyngitis were later affected by acute glomerulonephritis (Duca et al. 1969, Barnham et al. 1983).

An international collection of these strains of S.zooepidemicus isolated from human and animal infections were studied collaboratively to develop a system which could aid epidemiological studies. The various parts of this study were biochemical identification and serological typing (A.Efstratiou, Central Public Health Laboratory, London, U.K.); bacteriocin typing (J.Tagg, University of Otago, New Zealand); bacteriophage typing (S.Skjold, University of Minnesota, U.S.A.) and sensitivity to antibiotics (M.Barnham and G.Cole, Friarage Hospital, North Yorkshire).

The API 20 STREP system was useful in the identification of the organisms but was less helpful as a biotyping tool alone, as the majority of strains produced the same profile number, 4463607 (namely aesculin hydrolysed; beta glucuronidase formed, phosphatase and leucine aminopeptidase detected; arginine hydrolysed and ribose, sorbitol, lactose, starch and glycogen

fermented by these haemolytic strains).

The T-typing system achieved an 87% typability rate with human strains of S.equisimilis but it failed to type any of the S.zooepidemicus strains or indeed any animal isolate of group C. This illustrates the antigenic differences within this group because it was not possible to detect any T-protein antigens amongst animal isolates of Lancefield group C.

However, in the collaborative study, many of the S.zooepidemicus strains which showed the API profile number 4463607 could be distinguished by the combination of a bacteriocin method and a bacteriophage typing scheme which was specifically developed for S.zooepidemicus. DNA fingerprinting of the organisms also confirmed many of these distinctions (Skjold et al. 1987). The independent methods employed confirmed the similarities between strains and conversely demonstrated antigenic differences between S.equisimilis and S.zooepidemicus.

The apparent rarity of human infections due to S.zooepidemicus may also be attributable in part to the practice of identifying pyogenic streptococci to the level of Lancefield group only. Therefore, to gain more information on the nature and circumstances of S.zooepidemicus infections, perhaps isolates of group C streptococci isolated from man could indeed be identified to species level at least when infections are severe, invasive or followed by AGN, and above all when there appears to be an

association with animals, or when clusters of infections occur (Barnham et al. 1987).

4.2. INFECTIONS CAUSED BY LANCEFIELD GROUP G STREPTOCOCCI IN MAN

Since their first description by Lancefield and Hare (1935), the group G streptococcus has been recognised as part of the normal flora of the upper respiratory tract, skin, intestinal and female genital tracts. It has also been identified by some workers particularly within the last ten years, as the causative agent of invasive disease including endocarditis, septic arthritis, pharyngitis, puerperal sepsis, neonatal sepsis, cellulitis, pulmonary infections and otitis media. Several reports have linked many of these infections to an underlying malignancy in the affected person (Armstrong et al. 1970, Auckenthaler et al. 1983). In contrast other workers are still maintaining that these organisms remain an infrequent cause of disease (Rolston 1986).

The study was based on a total of 1211 isolates of group G streptococci. It was possible to subdivide serologically 82% of the isolates into 21 different serotypes. Again as with the group C streptococci, there were instances of cross-over between particular antigens of Lancefield groups A, C and G. This cross-over was epidemiologically useful as it increased the typability rate. The streptococci were isolated from various sites and were initially subdivided into 22 different T types. One type based on the strain 4860 was discarded from the scheme

because the vaccine strain was the only representative found. The largest collections examined were from the skin (40%), throat (20%), female genital tract (9%), infected burns (9%) and the blood (6%). These sites of isolation are perhaps not surprising as the organisms may exist as commensals on the skin (Sherman 1937), pharynx (Forrer and Ellner 1979) and female genital tract (Christensen et al. 1974). It was interesting to find that the original T types 7, 16, 20 and 21 first isolated by Griffith in 1934 from predominantly pharyngeal infections still seem to be associated with that site.

Septic arthritis due to Lancefield group G streptococci was not as uncommon as was previously thought. Reports in the early 1980s noted the rarity of septic arthritis caused by the streptococci of Lancefield group G (Lin et al. 1982). For example, the first report of this form of arthritis in Australia was in 1985 (March et al. 1985) and the first ever report in the literature was made in 1970 (Armstrong et al. 1970). The recent recognition and increased reporting of group G streptococcal arthritis may be apparent rather than real and could be related to the general availability of improved identification procedures for streptococci. The most common serotype encountered from cases of septic arthritis was type 20. The majority of the aspirate isolates received during this study (14 of 18) were reported to have been from cases of septic arthritis.

Group G streptococcal endocarditis is also being reported more

often. In general, three T types predominated amongst isolates from cases of endocarditis and septicaemia. These were serotypes 16, 301 and the complex pattern 7/302. Blood culture isolates of group G streptococci represented 6% of random isolates.

The clinical features of group G streptococcal bacteraemia in patients at a hospital in Nottingham were studied (Finch and Aveline 1984). Serotyping demonstrated five different T antigens. Four patients apparently acquired their infections after admission to hospital but nosocomial spread was not confirmed by the serotyping results.

Acute glomerulonephritis after infection with group G streptococci is apparently rare but has been reported in the West Indies (Reid et al. 1985) following infection of the skin.

Lancefield and Hare (1935) isolated group G streptococci from 5 of 855 ante-partum vaginal swabs and from the blood of a patient with puerperal sepsis, also infected with Staphylococcus aureus. They regarded the streptococcus as a secondary invader and reported that 'group A strains are probably the only hemolytic streptococci capable of causing definitive puerperal infection in the human species'. There are some later reports of isolation of group G streptococci from patients with puerperal sepsis or septic abortion (Colebrook and Purdie 1937, Hill and Butler 1940, Duma et al. 1969 and Baker 1974). In this study, isolations of group G streptococci from neonatal and maternal infections totalled

7%. As approximately 5% of women in labour harbour group G streptococci in their genital tract, their occasional involvement in puerperal sepsis, endometritis and neonatal infections is not surprising (Filkir and Monif 1979, Dyson and Read 1981). The presence of maternal or neonatal predisposing factors such as prolonged or difficult labour or the premature rupture of membranes increases the likelihood of infection (Baker 1974, Ancona et al. 1979). There were many pairs of isolates from mothers and babies. Common serotypes were 7/302, 20, 21, 301 and 308. Serotype 308 was established using strains isolated from an outbreak of infections of episiotomy wounds in a maternity unit.

As with Lancefield group C streptococci those group G strains isolated from animals could not be serotyped. This finding could explain the rarity of colonisation of humans with animal strains, if the T-protein antigen were an adherence factor.

There were approximately 50 clusters of cases examined which represented possible outbreaks of infection caused by Lancefield group G streptococci. Again, the diseases had some similarities to those caused by group A streptococci. These included strains from burns units, detention centres, maternity units and foodborne infection. The most common clusters were of skin sepsis and pharyngitis. Strains of a single serotype, namely 305, were isolated over one year from infected leg ulcers and the environment at an outpatient ulcer clinic in a London hospital. This constancy of type is consistent with an outbreak of

hospital-acquired infection. A possible vehicle of transmission appeared to be the skin flakes from the infected ulcers of these patients.

The largest single study on skin isolations of group G streptococci was in a London burns unit. Over the four year study period, 21% of patients admitted to the unit became colonised with group G streptococci and 15% with group A. The typing scheme established that certain serotypes occurred in the unit at different periods of time. This was evident at the start of the outbreak with the presence of one serotype (301) being associated with failure of skin grafts amongst several patients. Some 3% of patients admitted died soon after admission or at a later date from septicaemia and group G streptococci were isolated from the blood. Historically, the predominant streptococcus in burns units has been <u>Streptococcus pyogenes</u> (Lancefield group A). Some 40 years ago it was reported to be present in over 75% of admitted burns (Colebrook <u>et al.</u> 1945) although now it is closer to 4% (Lawrence 1985).

The most common outbreaks of group G infections studied were those of pharyngitis. This is reflected by the large numbers of throat isolates received. Asymptomatic throat carriage of group G streptococci occurs in up to 23% of humans (Hill <u>et al.</u> 1969). When present, symptoms of pharyngitis range from mild upper respiratory tract infection to severe exudative pharyngitis further complicated by lymphadenitis and fever. Acute rheumatic fever following group G streptococcal pharyngitis has not been described

but the occurrence of acute poststreptococcal glomerulonephritis in the West Indies has been suggested (Reid et al. 1985). Apparent outbreaks of infection were examined from closed institutions (detention centres, residential schools and army camps both in the U.K. and overseas). Asymptomatic carriage rates were also studied in one institution and was found to be 10%.

Foodborne outbreaks of pharyngitis caused by either Lancefield groups A, C or G streptococci are relatively rare. In this study, however, two separate foodborne outbreaks of group G pharyngitis were examined. Both outbreaks occurred overseas, one was in the U.S.A. (Stryker et al. 1982) and one in Israel. The former presumed outbreak occurred in Florida at a convention where 72 of 231 delegates interviewed became ill. Group G streptococci were isolated from the throats of 10 of 16 persons with pharyngitis and 1 of 41 persons without pharyngitis. The epidemiological data confirmed by the finding of the serotype 301 in all strains examined indicated that this outbreak of pharyngitis was caused by group G streptococci and that it was foodborne. All persons who had apparently attended the luncheon and had become ill had eaten a chicken salad at the luncheon. The sheer explosiveness of the outbreak suggested it had a common source. In the second foodborne outbreak, strains were available from pharyngitis cases, kitchen workers and from food samples. The group G streptococci from this outbreak could not be serotyped with the set of antisera available. It may therefore represent another T-type antigen.

Outbreaks of puerperal fever caused by group G streptococci have not been described until very recently (Haynes et al. 1987). The strains from this outbreak were examined and nine of ten cases carried strains of the serotype 7/302. The isolate obtained from the last case was T-type 21, but this patient was admitted after disinfection procedures had been introduced in the unit. All other patients used douches in the bathroom without prior disinfection although general cleaning of the douches with detergent was performed. When these were disinfected the outbreak ended abruptly. Environmental isolates from the bathrooms used by these patients yielded also the same serotype. A year previous to this outbreak, there was a cluster of infections in the same maternity unit where group G streptococci of serotype 7/302 were isolated from 11 of 12 cases of puerperal sepsis. This outbreak was cleared without the source being found. There were four additional fairly large clusters of infection in maternity units caused by Lancefield group G. A new serotype, type 308 was established from a maternity unit outbreak in one hospital. In all instances the typing confirmed that an outbreak had occurred and in one instance environmental isolates from the bathroom used by the patients yielded the same serotype. The fact that no other pathogen was isolated from all these patients, provides useful evidence that this was puerperal fever caused by group G streptococci. In these outbreaks none of the babies born to infected mothers showed evidence of invasive disease. Neonatal sepsis due to group G has been reported (Baker 1974) but is relatively rare. There were instances of paired isolates, where

group G streptococci were isolated from mothers and babies. In one instance, the mother carried a group G streptococcus serotype 20 in her vagina and the baby was subsequently colonised, however, neonatal sepsis was not observed. These four outbreaks drew attention to the possible significance of group G in the genital tract of parturient women.

Two hospitals, one in the south of England and the other in Scotland provided random isolates of group C and group G streptococci for a typing survey. Typability rates for the 'large colony' strains from both hospitals were reasonably similar being 83% for the south and 86% for the north, with a wide range of type distribution. The largest groups of isolates examined were from the throat, skin and genital tract. Many of the throat strains from Scotland belonged to the species S.milleri (23% with the group C antigen and 4% with the group G antigen). None of the isolates from the south belonged to the species S.milleri. Strains from the south were grouped and biotyped by the laboratory prior to submitting them for serotyping whereas all strains sent from Scotland were only serologically grouped. Certain serotypes appeared to predominate within each hospital, but there was no apparent evidence to suggest that any outbreak had occurred.

A brief antibiotic study involving 404 strains of group C and group G from sporadic non-related sources indicated and confirmed previous reports on the prevalence of erythromycin resistance in these organisms (Barnham and Cole 1986). They found that 1.4% of

group C and 1.6% of group G were resistant to erythromycin. In this study, the degree of resistance appears to be slightly higher with 2% of group C streptococci and 4% of group G exhibiting erythromycin resistance. MIC tests on a few resistant isolates of group G streptococci were performed by the Antibiotic Reference Laboratory, Central Public Health Laboratory, London. They showed erythromycin resistant cultures have an additional resistance to clindamycin. In contrast erythromycin resistant group A streptococci do not exhibit resistance to clindamycin. Erythromycin resistance was not apparently associated with a particular serotype, whereas tetracycline resistance was found in all strains carrying the T-complex pattern 7/302.

The biochemical identification of group C and group G streptococci has traditionally relied upon the fermentation of carbohydrates, particularly trehalose, lactose, raffinose and aesculin. These substrates were included in a recent scheme for the identification and subdivision within the group G streptococci. Clark et al. (1984) modified the scheme described by Biberstein and co-workers (1980) which was introduced solely for strains of animal origin. They (Clark et al. 1984) subdivided the species into eight biotypes based on the use of these four carbohydrates supplemented with enzyme tests. Their aim was to distinguish human from bovine isolates. Most of their human strains (42%) fell into their biotype 3 group which they defined as fermenting only trehalose and aesculin. The human strains of Lancefield group G that carried T-protein antigens were consistently aesculin and trehalose

positive in tests carried out with the API 20 STREP system. All (100%) therefore, from this study belonged to Clark's biotype 3 category.

The study of enzyme profiles seemed to be of greater value. The API ZYM system was used as a first step in the subdivision of the group C and group G streptococci. Then, from a battery of a total of 80 enzyme tests, nine reactions emerged as possibly useful. These nine substrates apparently differentiated between all species of Lancefield group C and group G streptococci and also between human and animal isolates that currently are placed within the same species. Human isolates of group G streptococci formed L-prolyl-L-arginine arylamidase and β-glucuronidase while the animal strains gave positive reactions for chymotrypsin and β-galactosidase. The differentiating enzyme for the human and animal isolates of group C (S.equisimilis) was α -L-glutamate arylamidase which was produced only by the human strains.

4.3. THE VIRULENCE OF LANCEFIELD GROUP C AND GROUP G STREPTOCOCCI

The T-protein antigens have proved useful markers for the study of apparent outbreaks of infection but they have not offered sufficient discrimination for a detailed epidemiological investigation nor are they likely to be involved in virulence. One of the characteristics of the streptococci of Lancefield group A is that they will survive and multiply in fresh normal human blood. The generally accepted explanation is that these organisms

resist phagocytosis by the presence of an M protein. These antigens can also be used as epidemiological markers supplementing the information obtained from the identification of T-protein antigens.

M-protein antigens have been described in Lancefield group C and group G streptococci (Maxted and Potter 1967, Lawal et al.1982). Some twenty years ago Maxted and Potter (1967) reported the presence of type 12 M antigen (which was originally isolated from group A streptococci and is a common serotype within that group) in three group G strains isolated from the throat and skin sores of children in the West Indies. Very little has been learned since then about the presence and distribution of such proteins in group C and G streptococci and their clinical significance. Lawal et al. (1982) reported that some strains of group G streptococci in Nigeria resisted phagocytosis in human blood. These strains however were not examined fully to determine whether they possessed other M proteins ordinarilly recognised in group A streptococci and were not apparently investigated to assess their relationship to disease.

The ability of human isolates of group C and group G streptococci, to survive and multiply in normal human blood was initially investigated with the stock vaccine strains used in the production of T-antisera. The strains were from a variety of sites and three were representatives from outbreaks of infection. None was from serious systemic disease. These streptococci exhibited varying

degrees of survival in the blood of human donors. Some did not survive at all. These initial results suggested that perhaps one half of all human strains of group C and group G streptococci did not possess an M-protein antigen or that there is a larger proportion of glossy variants than in group A streptococci. Alternatively, antibodies to the M protein may be widely distributed within the population facilitating the opsonisation and killing of the bacterial cells. Subculture of the survivor colonies and their exposure to further bactericidal tests showed that a streptococcal population with enhanced ability to survive and multiply in normal human blood had been selected. As the survival rate in bactericidal tests of streptococci isolated from either asymptomatic carriers, relatively mild infections and non-invasive disease appeared to be varied, isolates of group C and group G from blood cultures of patients with systemic disease were investigated. All such strains of streptococci tested survived in the blood of four human donors with increased counts ranging from 2 to 200-fold.

Freshly isolated strains of Lancefield group A, almost without exception, possess M-protein antigens, at least as judged by resistance to phagocytosis in fresh normal human blood but group C and group G streptococci are more heterogeneous. Bisno et al. (1987) have demonstrated by electron microscopy that seven group G strains possessed surface fibrils similar to those present in M-positive group A streptococci. These appendages however, were said to be sparse in two group G laboratory stock strains examined.

These findings seem to support the results presented here, that strains from systemic disease possess M-protein antigens whereas strains from less challenging infections or from carriers are variable in their possession of such factors. It may therefore be possible to distinguish major sub-groups within both of these Lancefield groups, those behaving in man as commensals and those organisms with properties associated with virulence.

Previous workers have noted the association of a 'non type-specific' cellular protein, the M-associated protein (MAP), with the M-protein antigen. In the Lancefield group A streptococci MAP has been identified as a non-specific component of the M-protein molecule itself. MAP has been identified in group C and group G streptococci (Lawal et al. 1982). The possibility was tested in this study by examining the relationship of MAP to survival properties in blood. Only one half of the strains tested reacted with one or other of two donor sera to titres of greater than 1 in 40. The third serum used in these tests was not active at all, therefore it is assumed that this serum is possibly devoid of complement-fixing antibodies. Overall, the results obtained indicated inconsistent relationships between reaction with sera containing antibody to MAP and survival in blood. Some four of nineteen strains tested exhibited very low MAP titres and survived in blood, ten strains survived in blood and reacted with MAP antibody, two did not survive in blood and neither did they respond to the MAP test and finally three strains which produced a significant MAP titre did not survive in blood. It is possible

that there may be several immunologically distinct varieties of MAP in these streptococci. If so these could be detected by testing with an extensive panel of normal sera. This therefore, could be another difference from strains of group A streptococci which usually produce one or other of two MAP antigens (Widdowson et al. 1971).

The survival and multiplication of these organisms in normal human blood appears to be a more direct indicator of virulence and was preferred for this reason. Testing for antibodies to MAP as an indicator of recent infection by group C or group G streptococci is a possibility; as has been done with group A streptococci (Widdowson 1980). An anti-hyaluronidase test (AHT,C/G) specific for group C and group G streptococci has been developed (Hallas and Widdowson 1982) but titres in these tests are lower than those developed for the equivalent, but antigenically distinct enzyme formed by group A streptococci. It is possible that those streptococci that do not contain large amounts of MAP but which survive and multiply in blood may possess other antiphagocytic factors such as has been suggested for some group A streptococci (Becker et al. 1973).

Candidate strains from blood cultures were tested as vaccine strains for the production of opsonic antisera. Whole cell vaccines have always been used for the preparation of M-typing sera for S.pyogenes. Following similar protocols, fourteen different possible M-typing sera were prepared. These sera produced clear

precipitin lines in double gel diffusion against acid extracts of the vaccine strains. All of these sera bar two were opsonic in an indirect bactericidal test (Maxted 1956). Such precipitating and opsonic rabbit antibodies were stimulated only by those streptococci possessing the ability to grow in fresh normal human blood. These results suggest the presence of M-like proteins on the vaccine strains. The exceptions gave rise to precipitating but not opsonic antibodies and were group C strains of serotypes 7 and 308. It has been demonstrated with group A streptococci (Fischetti et al. 1976) that some fractions of M-protein extracts produce precipitating but not opsonic antibodies; presumably the rabbit responded to different epitopes. A different form of variability was illustrated amongst some strains (four) of type 16 isolated from the blood or throat of different patients. Extracts of strains from both sources yielded precipitin lines in gel diffusion tests against specific opsonic antisera, but strains from blood cultures survived and grew in fresh normal human blood whereas strains from the throat were inconsistent. This variability could also be demonstrated amongst other serotypes from different sources. This could be explained by perhaps differences in the amount of M protein formed by different strains. Some M-protein positive strains of group G streptococci have an entire coating of fibrillar material whereas electron micrographs on supposedly M-negative variants showed only sporadic appendages (Bisno et al. 1987). Therefore, the protein may not be present as a uniform layer and perhaps binding sites are exposed on the cell surface which are specific for a particular membrane receptor on

the polymorph thus leading to ingestion and killing of the streptococcus by the phagocyte. Alternatively, these strains may be coated with a substance (a cationic surface protein) which promotes opsonisation, whereas M-positive strains may mask this particular substance, thus opsonisation is inhibited. Such a theory with involvement of the alternative complement pathway has been suggested (Bisno 1979 , Ginsberg et al. 1982).

It has been shown with group A streptococci that M-positive strains adhere more readily to human epithelial cells than do M-negative variants (Ellen and Gibbons 1972) and that lipotechoic acid (LTA) promotes such adherence (Beachey and Simpson 1982). Both LTA antigen and M protein can be detected on the cell surface as hair-like appendages by electron microscopy (Beachey and Ofek 1976). The notion that LTA rather than M protein plays a central role in the adherence process is based on the finding that streptococci treated with a mild pepsin digestion lose their M proteins but remain rich in LTA, retain their protrusions and adhere to epithelial cells just as well as untreated bacterial cells (Beachey and Ofek 1976). Possibly, therefore loss of these organisms from the throats of asymptomatic carriers may depend on the production of antibodies to LTA. Therefore, the mechanism involved in asymptomatic carriage and colonisation by group C and group G streptococci could conceivably also be related to LTA. This substance on the cell surface could be involved in the attachment of organisms to lipid binding sites of fibronectin molecules present on human oropharyngeal cells (Beachey and Ofek

1976). Perhaps in this instance T-protein antigens may play a role in adherence, and the proteins perhaps bind LTA on the surface in such a way which enables the streptococci to combine with epithelial cells of the host.

It is often stated that among the Lancefield group A streptococci, protection against infection is dependent on the presence of type specific antibodies to the M protein (Lancefield 1959). Guirguis et al. (1982) however found that the presence of type specific antibody did not protect against pharyngeal acquisition of organisms of the same serotype. Opsonic antibodies may therefore be important only in the prevention of invasive infections or other virulence mechanisms may determine colonisation in the throat.

It has also been suggested by Fischetti et al. (1986) that considerable differences in size can be detected in the M protein of Lancefield group A streptococci. These differences were found in proteins both within and between different serotypes. They isolated three independent M protein deletion mutants. By studying pepsin extracts of the various proteins they concluded the deletions were restricted to the section of the M-protein molecule distal to the cell wall surface in which sequence repeats are found. The simplest explanation of these changes is that there has been recombination between repeat regions of the genes controlling M-protein production. These changes appeared to be of importance in determining the survival of the organism in fresh normal human blood. Thus the heterogeneity detected amongst group

C and group G streptococci in this study could conceivably also be the result of differences within the corresponding region of DNA.

4.4. THE NATURE OF THE PROTEIN ANTIGENS

The study of the T- and M-protein antigens provided information which could be of value in the production of typing sera for strains of group A as well as group C and group G. Rabbit antisera to streptococcal proteins and carbohydrates have been prepared traditionally (Lancefield 1928a, 1928b) by intravenous inoculation with whole cell vaccines. Antisera to whole cell vaccines nearly always contain antibodies to the Lancefield group antigen of the vaccine strain. Two or more absorptions with cells of a heterologous type are required to remove the group antibody before the sera can be employed routinely. The T-protein antigens of Lancefield group A streptococci can be extracted with trypsin (McLean 1953) and a modification of this method allowed the preparation of immunogens that did not induce group antibody responses (Efstratiou 1985). The essential modification of McLean's method was the filtration of the trypsin extract. Initially, it was found that if rabbits were bled prior to week 4 of the inoculation schedule, group polysaccharide was not present. But, the group antibody was often detected at a later stage. Rhamnose is a component of the cell walls of streptococci of the Lancefield groups C and G (Curtis and Krause 1964) and it was detected in both the filtered and unfiltered T-protein extract albeit at lower levels than in intact cell walls. The presence of

particulate matter in the unfiltered extract was demonstrated by electron microscopy and the group antibody response in rabbits was associated with the presence of this material. Group antigens could not be detected in either filtered or unfiltered extracts by immunodiffusion tests against Lancefield grouping sera. This could suggest first that less material is required for an antibody response than for the development of a precipitin line or second, that the fraction containing rhamnose may not have diffused through the gel.

Pepsin extracts of group A streptococci have been used for the production of M antisera. Such vaccines were unexpectedly effective in stimulating antibodies to the T proteins of Lancefield groups C and G.

The titres induced in rabbits with the isolated T-protein antigens were lower than those produced with whole cell vaccines. The protein content of whole cells is perhaps greater or the cell wall mucopeptide may act as an adjuvant. Little has been written about the preparation of T-typing antisera for streptococci. Published work has centred upon the use of trypsinised whole cell vaccines for Lancefield group A streptococci. The use of isolated protein material has proved successful for providing typing sera for the group C and group G streptococci. The work should now be extended to the group A streptococci. As these T-protein precipitates are only partially purified and antibodies to trypsin were also produced it may be advantageous to purify further these proteins

by, for example, fast protein liquid chromatography.

There have been no detailed studies of the structure and composition of streptococcal T proteins. Detailed analyses were beyond the scope of this investigation but a limited study was made. The first workable method for the extraction of T protein was devised by Pakula (1951) and is based on an initial digestion of the bacterial cells with trypsin. The procedure in its original form and when modified yielded crude preparations contaminated with trypsin and probably other proteins. Chromatographic procedures were applied to attempt the separation of the T protein from trypsin. The methods employed included gel filtration, ion exchange chromatography and hydroxylapatite chromatography. Johnson and Vosti (1977) demonstrated a purification procedure for the group A T-protein serotype 1 by hydroxylapatite chromatography. This however was not successful for group C and group G T proteins, perhaps indicating a difference in composition. The presence of trypsin however did not apparently affect the use of these protein extracts for serum production but its presence was undesirable during biochemical analyses. Some rabbits developed antibodies to the enzyme and these sera were used to free the T-protein preparations of trypsin by means of affinity chromatography.

Purification by gel filtration on Sephadex G75 followed by affinity chromatography on cyanogen bromide activated Sepharose 4B coupled to either trypsin antibodies or a soya bean trypsin inhibitor

preparation resulted in a electrophoretically homogeneous fraction of T protein. This was detected as a single protein band on polyacrylamide gels scanned by laser beam densitometry and a single precipitin line in gel diffusion with the antiserum specific for that T protein.

The molecular weight of the extracted T protein as determined by polyacrylamide gradient gel electrophoresis and PAGE with the immunological location of the protein by immunoblotting was estimated to be within the range of 26,000 to 28,000 daltons. It was found to be a small weakly electromobile protein with an isoelectric point of approximately pH 4.5. The purified fraction was immunologically active. The molecular weight values were similar for both group C and group G proteins. Further analyses would be useful to test for molecular similarities. There have been two reports which gave an estimated molecular weight and amino acid composition for the T proteins of group A, serotypes 1 and 12. The preparations made by Johnson and Vosti (1977) indicated that the type 1 antigen consisted of subunit size isomers and contained glycine, aspartic acid, glutamic acid and serine as the five predominant amino acids. They did not give an estimation for the molecular weight of the T protein. However, Ludwicka and Kloczewiak (1978) stated that their T-protein preparation of serotype 12 yielded a molecular weight of approximately 38,000 daltons. Both groups of workers reported iso-electric points at pH 4.5 for these proteins.

Attempts have been made in the past to isolate the group-A streptococcal M protein. One successful method was based on extraction of the protein with a proteolytic enzyme and purification by ammonium sulphate precipitation, ribonuclease digestion, ion exchange chromatography and isoelectric focusing (Johnson and Vosti 1968, Fischetti et al. 1976, Jones and Fischetti 1987). Purification procedures used in this study included initially, the isolation of the protein by crude acid extraction and purification by fractional precipitation with ammonium sulphate. Precipitates obtained at 30% and 60% saturation with ammonium sulphate were found to be devoid of detectable M-protein antigens as judged by failure to induce antibodies. The protein contents of the fractions were low when compared to the yields from other procedures.

Affinity chromatography had been useful in earlier experiments for the purification of the T protein, the technique was used for the preparation of an M-protein extract devoid of the group polysaccharide but with potential immunogenicity in rabbits. In these experiments, crude acid extracts were separated on affinity gels to which had been coupled group polysaccharide antibodies. The M-protein substance was separated from the group polysaccharide by this method. Animals were subsequently inoculated but none of the fractions induced an antibody response. These fractions did however exhibit precipitin lines in immunodiffusion tests with antisera prepared from whole cell vaccines. These findings confirm those of other workers (Barkulis

and Jones 1957) that acid extracted M protein does not induce an antibody response in rabbits.

The sensitivity of streptococcal M-protein extracts to proteolytic enzymes was first described by Lancefield in 1928 for Lancefield group A streptococci. She observed that by altering digestion time and enzyme concentrations, different moieties of M-protein antigens could be extracted. These were described by her as type specific and non-type specific. However, no further attempt was made to determine the purity of the material after such digestion. Beachey and his colleagues (1974) found that controlled proteolytic digestion would release an M-protein fraction that is still immunogenic when used with an adjuvant. The data presented here suggests that the M-protein antigens of Lancefield group C and group G streptococci have obvious similarities to the pepsinised material handled by Beachey. Pepsin extracts were found to be richer in type specific M protein than conventional acid extracts. They were, as reported by Beachey et al. (1974) for the group A streptococci, free of polysaccharide but in contrast to their findings did contain T protein because a T-antibody response occurred in the immunised animals. The absence of the group polysaccharide overcame the need for absorption of the typing sera. The immunogenicity of pepsin extracts when combined with an adjuvant was confirmed in animals. In such preparations an aluminium hydroxide gel, as an adjuvant, induced antibodies to the T protein but not the M protein. An oil based emulsion, Freund's complete adjuvant containing a pepsin extract stimulated both M and

T antibodies. For Freund's complete adjuvant it has been suggested that the cell wall lipids and mucopeptide of the mycobacterium present in the emulsion are the active ingredients (Stites et al. 1982). This may explain the stimulation of group, M and T antibodies when whole cell vaccines are used because of the large amount of the mucopeptide of necessity present.

Analyses of pepsin extracts of both group C and group G streptococci on SDS PAGE gels gave several distinct bands especially within the molecular weight range of 20,000 to 40,000 daltons. Western blot analysis with polyclonal rabbit sera was then employed. For the first immunoblot experiment an antiserum specific for T protein was used. This yielded a distinct band with an approximate molecular weight of 27,000 daltons. The value was in agreement with previous determinations on trypsin extracts. In the second experiment, an antiserum with both T- and M-protein antibodies was employed. Two bands were detected, one in the region of 27,000 daltons and thus presumably T protein and the other within the 34,000 daltons region. This latter is similar to the molecular weight estimation for Lancefield group A streptococcal M proteins, also extracted with pepsin, of 35,000 daltons (Beachey et al. 1977). These molecular weight determinations are also in accord with other reports. Jones and Fischetti (1987) found that the molecular weights for their group G streptococcal M-protein preparations were within 30,000 to 43,000 daltons. Bisno et al. (1987) reported similar estimates of 31,000 to 45,000 daltons.

In conclusion, the data presented in this study indicates the similarities of Lancefield groups A, C and G streptococci and an even closer relationship between groups C and G of human origin, with particularly in their cell wall protein antigens and their ability to cause both systemic diseases and outbreaks of infection. These properties were tested by the application of the serotyping scheme developed. Parker et al. (1976) described three major criteria for the evaluation of a serotyping scheme. They are typability, reproducibility and discrimination. It may be concluded that this system offered a useful typability rate with 87% of group C and 82% of group G being agglutinated. Secondly, reproducibility, this has two meanings. One interpretation is laboratory reproducibility which is the degree to which different laboratories typing the same strain achieve the same result. This problem has not yet been addressed. Another interpretationof reproducibility is more related to the stability of a particular serotype. Instability has been described, for instance in bacteriophage patterns in a collection of group B streptococci that were epidemiologically related (Noya et al. 1987). The T proteins in contrast were found to be very stable antigens and exhibited a high degree of reproducibility amongst epidemiologically related strains. For example, the continuing outbreaks occurring at an ulcer clinic (Table 56) of a London hospital and at the Burns Unit of another hospital (Fig.35). Thirdly, discrimination which is limited by the size of the largest single group of representatives of a single serotype within the scheme. In this study the two

most common serotypes within group C, comprised 20 and 23% of the total serotype distribution, for group G, the two most common types comprised 13 and 18% of the total respectively. Greater discrimination could possibly be achieved by the introduction of a complementary typing scheme based on the M-protein antigens.

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Appendix 1

ENZYMES TESTED FOR:

1. L-tyrosine arylamidase
2. L-pyrrolidone arylamidase
3. L-phenylalanine arylamidase
4. L-lysine arylamidase
5. L-hydroxyproline arylamidase
6. L-histidine arylamidase
7. Glycine arylamidase
8. L-aspartate arylamidase
9. L-arginine arylamidase
10. L-alanine arylamidase
11. γ-glutamyltransferase
12. N-benzoyl-leucine arylamidase
13. S-benzyl-cysteine arylamidase
14. Methionine arylamidase
15. Glycyl-glycine arylamidase
16. Glycyl-phenylalanine arylamidase
17. Glycyl-proline arylamidase
18. Leucyl-glycine arylamidase
19. L-seryl-tyrosine arylamidase
20. Negative control
21. N-CBZ-arginine-4-methoxy-arylamidase
22. L-glutamine arylamidase
23. α-L-glutamate arylamidase
24. L-isoleucine arylamidase
25. L-ornithine arylamidase
26. L-proline arylamidase
27. L-serine arylamidase
28. L-threonine arylamidase
29. L-tryptophane arylamidase
30. N-CBZ-glycyl-glycyl-arginine arylamidase
31. β-alanine arylamidase
32. L-alanyl-arginine arylamidase
33. L-alanyl-L-phenylalanyl-L-proline arylamidase
34. L-alanyl-L-phenylalanyl-L-prolyl-L-alanine arylamidase
35. L-arginine-L-arginine arylamidase
36. α-L-aspartyl-L-alanine arylamidase
37. α-L-aspartyl-L-arginine arylamidase
38. α-L-glutamyl-L-glutamic arylamidase
39. α-L-glutamyl-L-histidine arylamidase
40. Glycyl-L-alanine arylamidase
41. Glycyl-L-arginine arylamidase
42. Glycyl-L-tryptophane arylamidase
43. L-histidyl-L-leucyl-L-histidine arylamidase
44. L-histidyl-L-serine arylamidase
45. L-leucyl-L-alanine arylamidase
46. L-leucyl-L-leucyl-L-valyl-L-tyrosyl-L-serine arylamidase
47. L-lysyl-L-alanine arylamidase
48. L-lysyl-L-lysine arylamidase
49. L-phenylalanyl-L-arginine arylamidase
50. L-phenylalanyl-L-proline arylamidase

51. L-phenylalanyl-L-prolyl-L-alanine arylamidase
52. L-prolyl-L-arginine arylamidase
53. L-seryl-L-methionine arylamidase
54. L-valyl-L-tyrosyl-L-serine arylamidase
55. N-benzoyl-L-alanine-4-methoxy arylamidase
56. N-CBZ-arginyl-4-methoxy arylamidase
57. N-CBZ-glycyl-glycyl-L-arginine arylamidase
58. N-acetyl-glycyl-L-lysine arylamidase
59. L-histidyl-L-phenylalanine arylamidase
60. L-lysyl-L-serine-4-methoxy arylamidase
61. Negative control
62. Alkaline phosphatase
63. Esterase (C4)
64. Esterase lipase (C8)
65. Lipase (C14)
66. Leucine arylamidase
67. Valine arylamidase
68. Cystine arylamidase
69. Trypsin
70. Chymotrypsin
71. Acid phosphatase
72. Napthol-AS-BI-phosphohydrolase
73. α -galactosidase
74. β-galactosidase
75. β-glucuronidase
76. α -glucosidase
77. β-glucosidase
78. N-acetyl-B-glucosamine
79. α mannosidase
80. α fucosidase
81-100. Esterases and osidases

Enzyme differentiation of human and animal isolates of Lancefield group G streptococci

API 20 STREP SYSTEM:

VP: Acetoin production

HIP: Hippurate hydrolysis

ESC: β-glucosidase

PYRA: Pyrrolidonylarylamidase

α-GAL: α-galactosidase

BGUR: β-glucuronidase

BGAL: β-galactosidase

PAL: Alkaline phosphatase

LAP: Leucine arylamidase

ADH: Arginine dihydrolase

RIB: Ribose

ARA: Arabinose

MAN: Mannitol

SOR: Sorbitol

LAC: Lactose

TRE: Trehalose

INU: Inulin

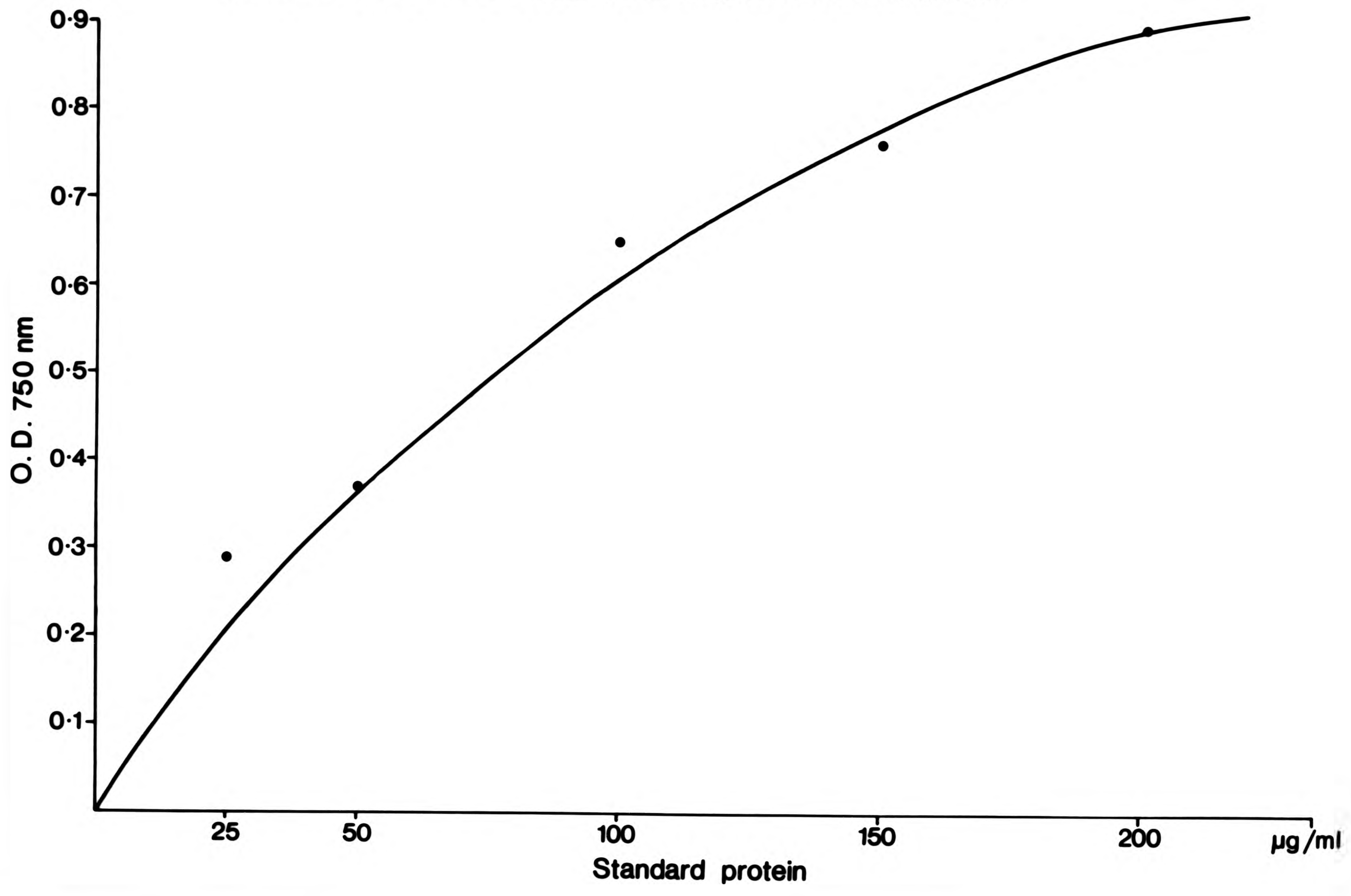
RAF: Raffinose

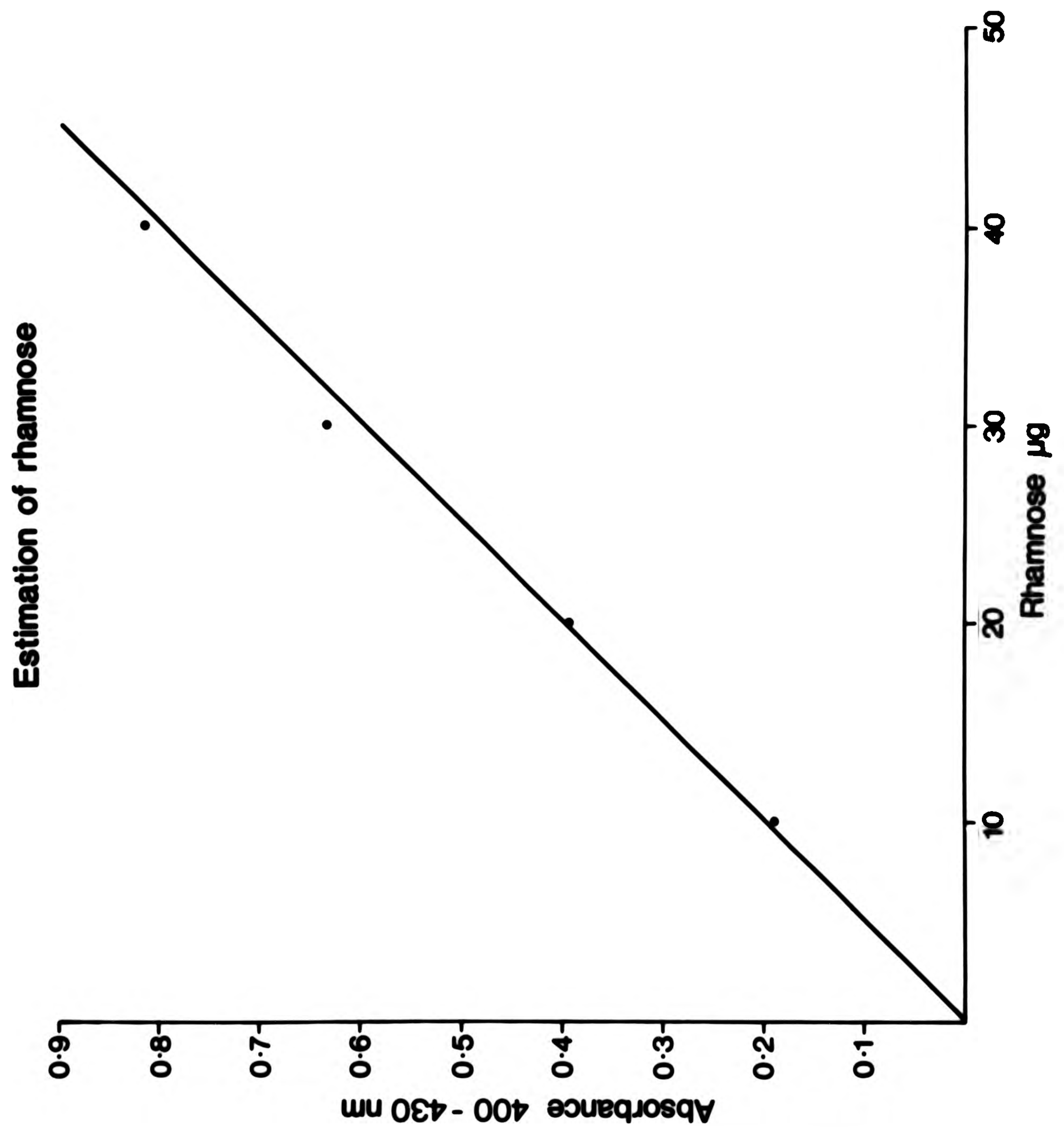
AMD: Starch

GLYG: Glycogen

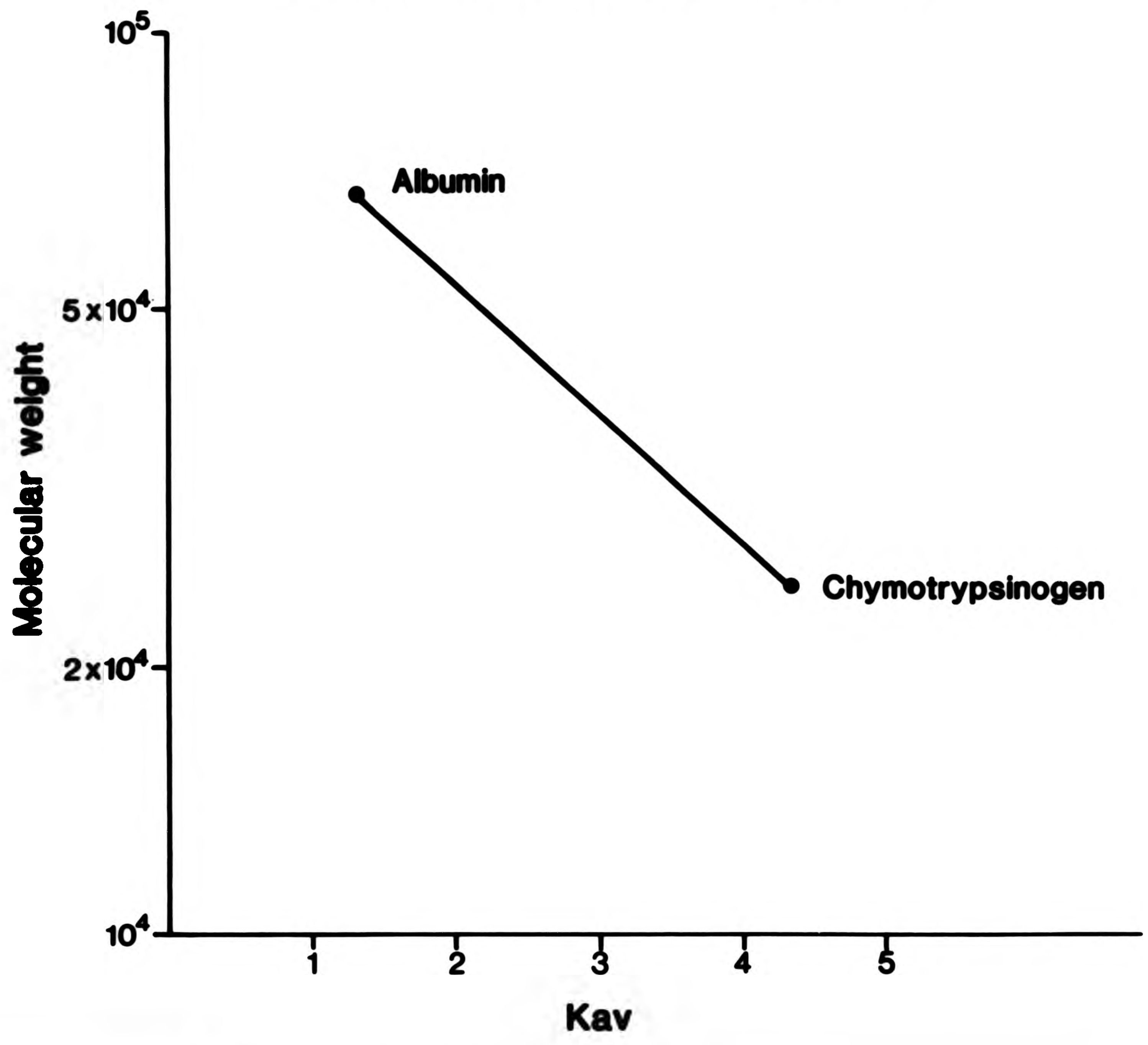
Appendix 2

Standard curve for the estimation of protein concentration

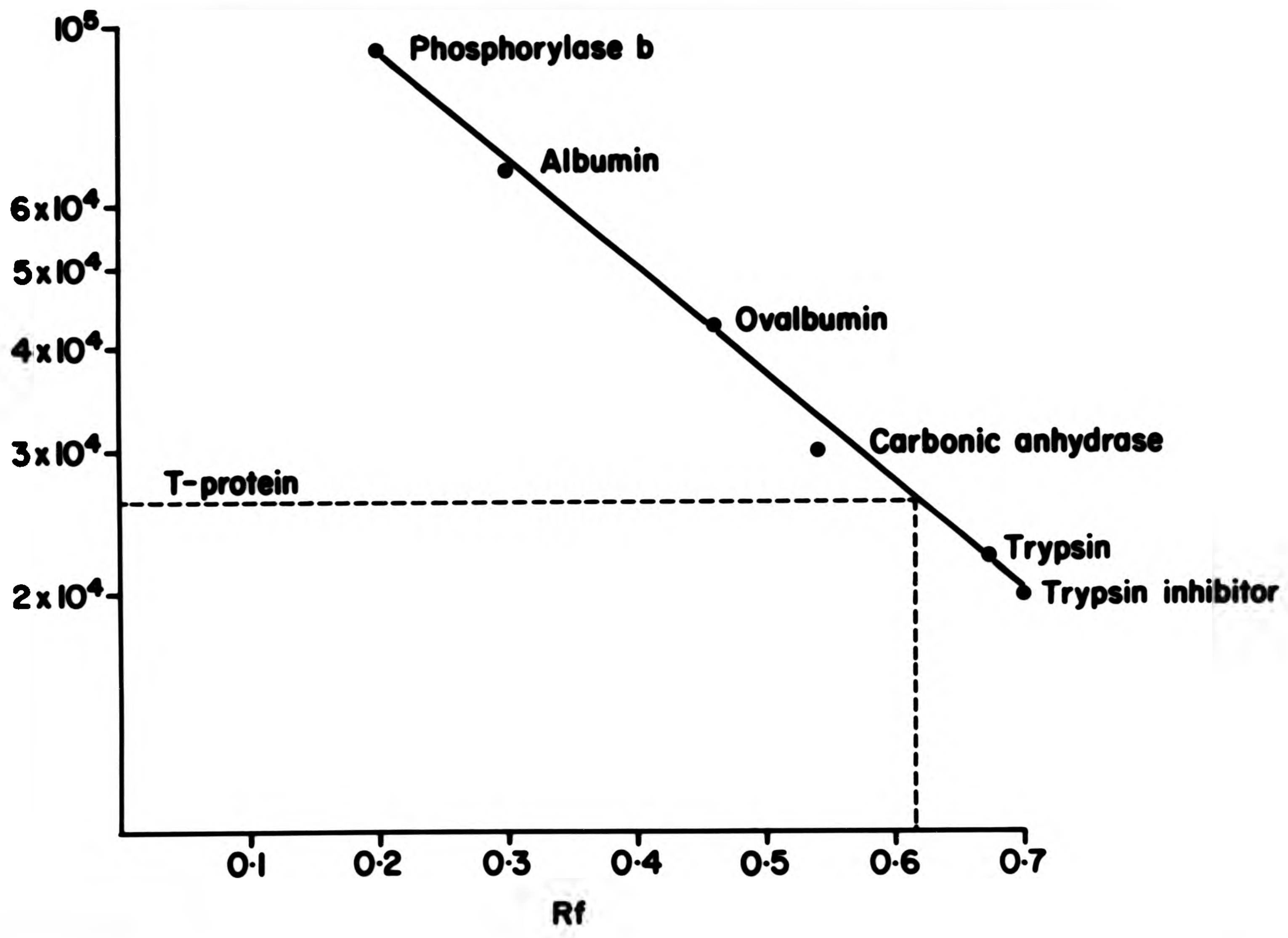



Estimation of rhamnose
Absorbance 400 - 430 nm
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
Rhamnose µg
10
20
30
40
50

Calibration curve for Sephadex G-75

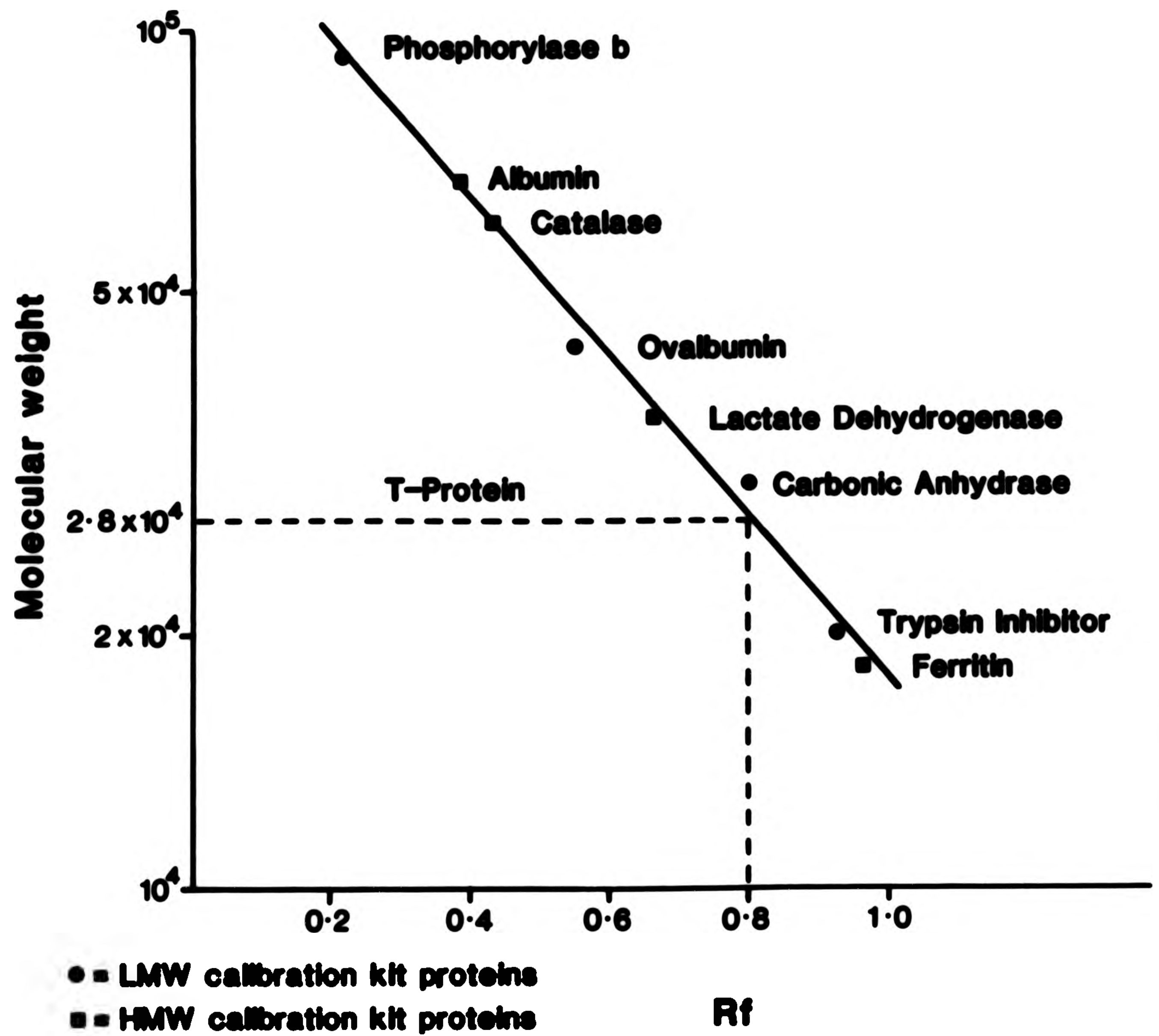


Calibration curve established with LMW markers and the location of T-protein on a high density gradient gel



I. Calibration curve for SDS-PAGE on 12.5% gels

II. Location of the T-protein



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The preparation of antisera to the T-proteins of Lancefield group C and group G streptococci

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Summary

A method for the preparation of antisera to the T-protein antigens of pyogenic streptococci is described. Sera produced by standard methods require absorption before they can be used for typing strains. The use of trypsin-extracted T-proteins as immunogens largely avoids the need for these absorptions. The antibody response to the partially purified material, although of a lower titer than a whole-cell vaccine, is type specific.

Key words: *Group C streptococci - Group G streptococci - T-proteins*

Introduction

Investigations of the spread of bacteria are made more precise by the use of typing schemes that subdivide species. The T-protein antigens of the pyogenic streptococci are sufficiently numerous to be used for this purpose and are the basis for one of the established methods for typing *Streptococcus pyogenes* (Lancefield group A). The method has been extended [1] and most human strains of Lancefield group C or group G streptococci carry one or other of 18 T-proteins specific for these two groups.

Whole-cell suspensions of representatives from both Lancefield groups C and G are excellent immunogens, and rabbit antisera have agglutinating titers ranging from 500 to 10000. The antisera nearly always contain antibodies to the Lancefield group antigen of the vaccine strain. Two or more absorptions with cells of a heterologous type are required to remove the group antibody before the sera can be used.

Methods have been described for the isolation and partial purification of T-proteins [2-4]. These methods were modified for this investigation in the hope that their use as immunogens would improve the preparation of antisera.

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Materials and Methods

Vaccine strains

Representatives of all types were used [1], after more detailed studies employing a strain of Lancefield group C, type 204 (NCTC 11629) and one of group G type 308 (NCTC 11630).

Preparation of whole-cell T-vaccines

Each strain was grown in 250 ml of Todd-Hewitt broth (Media Dept. C.P.H.L.) containing 1% (v/v) of a 10% (w/v) sterile saturated solution of Trypsin 1:250 (Difco) [3]. Incubation was at 30°C for 24 h to enhance the production of the T-antigen. The cells were harvested, washed and resuspended in 25 ml of phosphate-buffered saline (pH 7.8, 0.1 M saline, 0.2 M phosphate) containing 0.5% (v/v) trypsin solution and left at 22°C for 24 h. The cells were then washed six times in sterile physiological saline and finally resuspended in 17 ml of saline and 3 ml of a formalin solution (8% (w/v) formaldehyde in saline) and left at 22°C for 4 h. The formaldehyde content of the suspension was reduced by centrifuging the suspension at 4000 × *g* for 20 min and replacing half the volume of supernate with saline.

Preparation of isolated T-protein vaccines

Bacterial cultures were grown in 4 l of Nutrient Broth (Oxoid) supplemented with glucose, 0.5% (w/v) for 72 h at 22°C. The cultures were then heated to 70°C for 10 min to kill the cells, and then allowed to stand overnight. The clear supernate was siphoned off and the remaining suspension centrifuged at 4000 × *g* for 20 min, then washed twice in physiological saline. The cells were resuspended in 18 ml of 0.1 M phosphate buffer (pH 7.8) and 2 ml of a sterile saturated solution of Trypsin 1:250 (Difco) was added. The pH was increased to 8.2 with 1 M sodium hydroxide, and the mixture incubated at 50°C for 1 h. After digestion the suspension was centrifuged and the supernate filtered through a sterile cellulose acetate filter, pore size 0.45 μm. For some experiments the supernate was not filtered. The filtrate and unfiltered supernate were acidified to approximately pH 2.5 to precipitate the fraction containing the T-protein. The fluid was stored overnight at 4°C, centrifuged, the supernate discarded and the deposit dissolved in 2 ml of 0.1 M phosphate buffer (pH 7.8). The protein content determined by the method of Lowry et al. [5] was approximately 2 mg/ml. All extracts were then tested by double gel diffusion against group- and type-specific antisera.

Immunization procedures

A single schedule was used for each whole-cell vaccine. Each rabbit (New Zealand White) was given a primary intravenous injection of 1 ml of the whole-cell vaccine, and this was followed 2 weeks later by four injections, of the same dosage and by the same route, with intervals of one week between each.

For inoculation, the T-protein extract was diluted to 400 μg/ml with saline. Comparisons were then made using subcutaneous and intravenous injections of the T-protein preparation with and without the presence of an adjuvant, aluminium hydroxide gel (10% v/v of a 2% w/v gel, Serva).

The schedule for the intravenous inoculations of the isolated antigen was similar to the schedule described for the whole-cell vaccines. For the subcutaneous inoculations, two doses of 0.5 ml diluted T-extract were given in two sites as the primary dose. This was followed by a course of weekly vaccinations of a 1 ml dosage (0.5 ml in each of two sites) for 4 weeks, 2 weeks after the primary dose.

Absorption of the antisera

The antisera produced from whole-cell vaccines required absorption with equal volumes of packed bacterial cells prepared from an appropriate strain [6]. The absorptions were performed at 22 and 37°C for 24–48 h [1].

Preparation of bacterial cells walls

Bacterial cell walls of a representative of serotype 308 were prepared by washing cells that had been broken in a Mickle tissue disintegrator. In brief, the broken cells were washed thrice in sodium dodecyl sulphate (1% w/v). Differential centrifugation was then carried out to remove intact cells and finally the walls were washed thrice in distilled water and were then freeze-dried.

Rhamnose estimation

The rhamnose content of intact cell walls, and the filtered and unfiltered supernates following trypsin digestion but prior to acid precipitation, were estimated by the method of Gibbons [7].

Preparation for electron microscopy

Negative staining using a 3% phosphotungstic acid solution was performed on the cell walls, unfiltered and filtered extracts. Formvar-coated grids were used. The preparations were examined by electron microscopy on an AEI EM 6B operating at an acceleration voltage of 60 kV. The magnification used was ×30000.

Results

Titers

The antisera were tested for precipitation with the isolated T-antigens by double gel diffusion [8], and also by the agglutination of trypsinised whole cells [9]. The latter method is used in the routine T-typing of streptococci. The agglutination titers obtained with the partially purified protein were usually 8 or 16 (Fig. 1). In the first series of experiments the trypsin digests obtained from washed cells were not filtered (as described under Methods) and antibodies to the group polysaccharide were often detected during the fourth week of immunization. These antibodies did not develop if the supernate was filtered prior to acid precipitation of the T-protein (Fig. 1).

The agglutination titers obtained with the partially purified T-protein were lower, usually 8 or 16, than the titers obtained when whole cell vaccines were used. However, the antisera prepared from whole cells contained high titers of the group polysaccharide antibody, usually within the range of 5000 to 10000. Therefore, for removal of the group antibody, the antisera required heavy absorptions with packed streptococcal cells

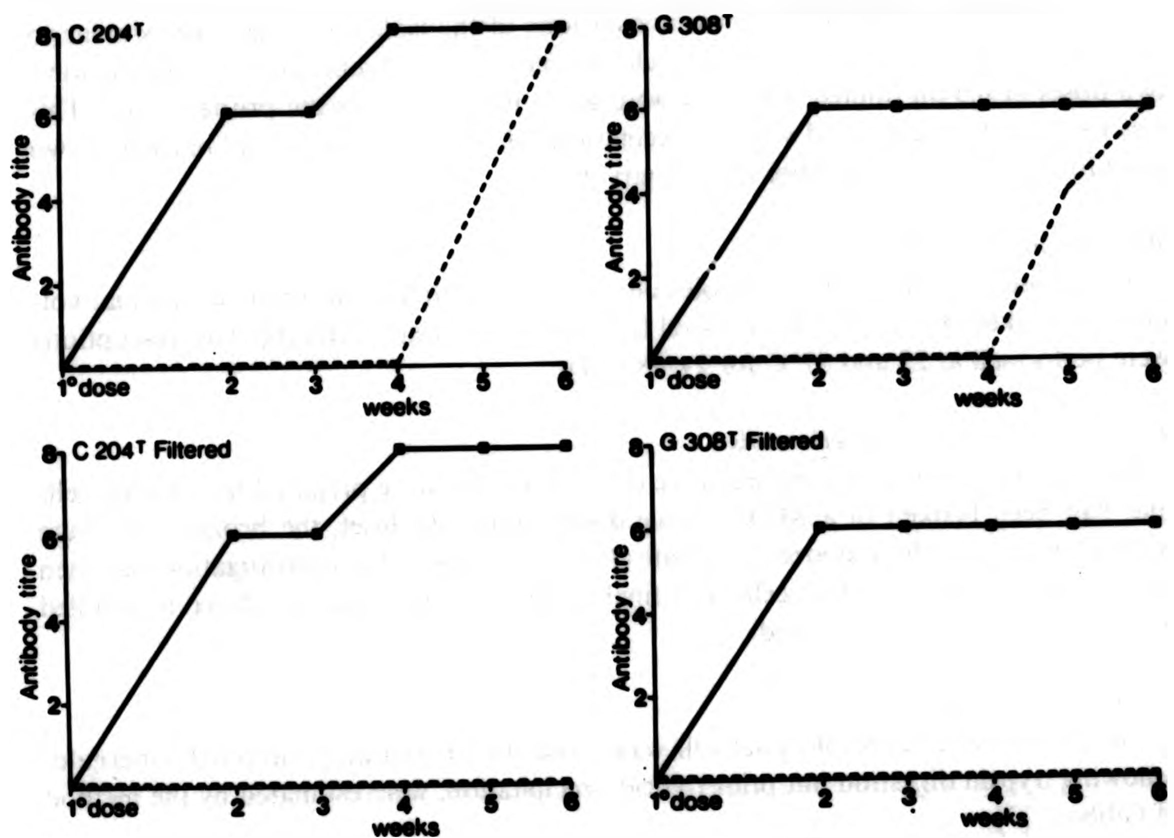

Fig. 1. Comparison of the antibody response in rabbits to the T-protein using filtered and non-filtered immunogens. ■——■, T-antibody response; - - - - -, group antibody response.

over a period of 48 h. The T-antibody titer from the absorbed antisera varied from 500 to 5000.

Antisera prepared from isolated T-proteins were always type-specific with titers of approximately 10. Group antibody was not present using the method described.

The use of different routes of inoculation did not lead to any consistent difference in titers and the schedule described was adopted.

Specificity

Antibodies to the Lancefield group antigens were not detected in sera prepared against filtered extracts but were always present in antisera prepared with whole cell vaccines, often with group agglutinin titers as high as 10000.

Differences were not found in the specificity to the T-proteins whether a whole cell vaccine or an extracted antigen was used.

Multiple reactions are occasionally found with particular strains, for example, agglutination with serotypes 7 and 302 or 301 and 305 are sometimes seen with representatives of Lancefield group G. There was no evidence that these reactions were more, or less, frequent with the semi-purified immunogen than with the whole cell vaccine.

Antibodies to trypsin could be demonstrated in some sera by gel diffusion tests.

When present, the titers were lower in the sera prepared using the extracted antigens and could only be detected in undiluted antisera. These sera are diluted 1 in 4 for routine use.

Rhamnose estimation

The amount of rhamnose per mg dry weight was determined for whole cell walls and the unfiltered and filtered supernates. The rhamnose content was found to be 1.0 and 0.8% of the dry weights of both the unfiltered and filtered extracts, respectively, and 12% of the dry weight of intact cell walls.

Electron microscopy

In the negatively stained preparations the cell walls were visualized as collapsed walls without adhering debris. Neither bacterial cells nor cell walls were found in the trypsin extracts, but granular material lacking a recognizable structure was seen in the unfiltered material.

Discussion

The production of antisera for typing streptococci is laborious but a very small volume, less than 0.05 ml, of each typing serum is used to serotype one strain. The serum produced by one rabbit, if stored undiluted will be sufficient for several years routine work. The absorption of streptococcal typing sera is more expensive in time and materials than the production of the original sera. Thus, 10 l of broth culture will produce sufficient packed cells for the absorption of 20 ml of antiserum. The group polysaccharide antibody in group C and group G antisera usually requires two separate absorptions at 22°C for 48 h. The avoidance of these lengthy procedures is the main advantage in the use of extracted T-antigens as immunogens.

The trypsinized cell walls of Lancefield groups A, C and G streptococci contain two major components, the mucopeptide matrix consisting of *N*-acetylmuramic acid, *N*-acetylglucosamine and amino acids; and the group specific carbohydrate which for Lancefield group G is mainly composed of rhamnose. Quantitative precipitin inhibition studies have shown that rhamnose markedly inhibited the group G precipitin reaction [10]. Rhamnose was detected in both the filtered and unfiltered extracts but at lower levels than in the intact cell walls.

The presence of particulate matter in the unfiltered extract was demonstrated by electron microscopy and the group antibody response in rabbits was associated with the presence of this material. Group antigens could not be detected in either extract by double gel diffusion against Lancefield grouping sera. But, these negative findings do not distinguish between the possibilities that, first, less material may be required for an antibody response than for the development of a precipitin line, or second, that the fraction containing rhamnose may have diffused poorly through the agar gel.

Little has been written on the preparation of T-typing antisera for streptococci. Published work has centred upon the T-antigen of Lancefield group A streptococci, where whole-cell vaccines have been used. The titers induced in rabbits to both the group and T-antigens in group A streptococci are lower than those obtained for the group C and group G streptococci.

There is an increasing awareness of the pathogenicity of group C and group G streptococci for man and the development of a typing scheme for these organisms should aid epidemiological studies.

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Characterization of *Streptococcus zooepidemicus* (Lancefield group C) from human and selected animal infections

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SUMMARY

We assembled an international collection of strains from sporadic and epidemic human infection with *Streptococcus zooepidemicus* (Lancefield group C) for laboratory study. Cultural and physiological characteristics of the isolates were determined, including biotyping with the API 20 STREP test kit and susceptibility testing with penicillin, erythromycin and tetracycline. The strains were examined for bacteriocin production and sensitivity and typed with a specially developed group-C streptococcal bacteriophage system incorporating a panel of 14 phages. Results of these tests gave useful discrimination between many of the strains: differences were shown between each of the major outbreak strains, including those complicated by post-streptococcal glomerulonephritis.

Serious group C streptococcal infection may be caused by *S. zooepidemicus* and isolates should be identified to species level; the application of a typing scheme such as this may help to distinguish epidemiological patterns of infection.

INTRODUCTION

Streptococcus zooepidemicus causes infection in a wide range of animals but it has been found rarely in man (Parker, 1983). The few reports of human infection include upper respiratory tract infection, cervical lymphadenitis, pneumonia, septicaemia, endocarditis and meningitis, usually in patients in close contact with

horses or drinking unpasteurized milk (Barnham, Thornton & Lange, 1983). It seems that the infection should, in general, be regarded as a zoonosis.

Human infection is often sporadic and may be complicated by post-streptococcal glomerulonephritis (PSGN) (Barnham, Ljunggren & McIntyre, 1986). Recent reports of outbreaks in communities where unpasteurized dairy products are consumed featured pharyngitis, complicated in a proportion of patients by PSGN (Duca *et al.* 1969; Barnham, Thornton & Lange, 1983), or severe invasive disease with a high mortality rate (Ghoneim & Cooke, 1980; Morbidity and Mortality Weekly Report, 1983; PHLS Communicable Disease Surveillance Centre, 1984, unpublished) although some patients at risk of infection remained well.

We have gathered together an international collection of strains of *S. zooepidemicus* from human infection, together with some associated isolates from animals. We present here the results of laboratory studies to show the characteristics of the isolates, and the development of a typing system based on bacteriocin, bacteriophage and biotyping tests. We hope that the application of this system will help in the investigation of future incidents.

MATERIALS AND METHODS

Collection of organisms. Isolates of *S. zooepidemicus* were collected from sporadic and epidemic human infections, and from related animal sources, as shown in Table 1. Organisms were isolated locally in North Yorkshire or kindly donated by doctors and laboratories as shown in the table. Index strains from the Halifax outbreak (isolate number 8a) and the Northallerton outbreak (10c) have been laid down in the National Collection of Type Cultures at the Central Public Health Laboratory, Colindale, code numbers NCTC 11854 and 11606 respectively. Altogether 46 isolates were assembled: 31 from human infection, 11 from animals and 3 from dairy products, plus 1 from a carrier (whether human or animal unknown) in the follow-up studies to the Romanian outbreak of 1968.

Isolates numbered 1 and 6 were from human infections related to horses (see the references in Table 1) and the series of isolates numbered 8, 9, 10 and 11 were from infections considered to be due to the consumption of unpasteurized cow's milk or its products. Isolate 7 was from a cat fancier with an infected finger who also kept poultry and donkeys. Series 13 was from an episode of bovine mastitis which did not lead to human infection. In series 8 and 13 isolates from horses on the farms were included in the collection as these animals were thought to be possible original sources of the bovine infection. Isolate 12 was from one of a litter of piglets that died of septicaemia in North Yorkshire while our study was in progress; there was no related human infection.

Acute PSGN was seen in man as a complication of infection with isolate numbers 5, 10a, 10c and 11a.

Colonial morphology. Organisms were cultured on Columbia agar (Oxoid Ltd., Basingstoke, Hants., code CM331) containing 5% defibrinated horse blood, incubated at 37 °C in air for 24 h and examined with a plate microscope.

Identification. The Lancefield group C antigen present in all strains was detected initially with the Streptex grouping kit (Wellcome Diagnostics, Dartford, Kent) and confirmed by testing acid extracts, in parallel with a reference strain, against

Table 1. *Origin of the study strains of* S. zooepidemicus

Isolate no.	Patient/ animal	Source	Date	Given by/reference
Sporadic infections				
1	Patient (neonate)	C.s.f.	1976–82	Prof. Zanen/Mulder *et al.* (1984)
2	Patient	Leg ulcer	Oct. 82	—/Barnham (1987)
3	Patient	Sore throat	Jan. 83	—/Barnham (1987)
4	Patient	Knee aspirate	May 85	—/Barnham *et al.* 1987)
5	Patient	Blood culture	July 85	Dr Lunggren/Barnham *et al.* 1987)
6a	Patient	Blood culture	Sept. 85	Dr Skirrow/Barnham *et al.* 1987)
6b	Horse	Tracheostomy	Sept. 85	Worcester VIC/Barnham *et al.* (1987)
6c	Horse	Tracheostomy	Sept. 85	Worcester VIC/Barnham *et al.* (1987)
7	Patient	Finger	Feb. 86	—/Barnham (unpublished)
Halifax (Yorkshire) outbreak				
8a	Patient	Blood culture	Mar. 84	Dr Edwards/PHLS CDSC (1984)
8b	Patient	Blood culture	Apr. 84	Dr Edwards/PHLS CDSC (1984)
8c	Patient	Blood culture	Apr. 84	Dr Edwards/PHLS CDSC (1984)
8d	Patient	Aneurysm	Apr. 84	Dr Edwards/PHLS CDSC (1984)
8e	Patient	Blood culture	Apr. 84	Dr Edwards/PHLS CDSC (1984)
8f	Patient	Blood culture	Apr. 84	Dr Edwards/PHLS CDSC (1984)
8g	Patient	Blood culture	Apr. 84	Dr Edwards/PHLS CDSC (1984)
8h	Patient	Blood culture	Apr. 84	Dr Edwards/PHLS CDSC (1984)
8i	Patient	C.s.f.	May 84	Dr Edwards/PHLS CDSC (1984)
8j	Patient	Blood culture	June 84	Dr Edwards/PHLS CDSC (1984)
8k	Patient	Necropsy	May 84	Dr Edwards/PHLS CDSC (1984)
8l	Cow T66	Udder	May 84	Leeds VIC/PHLS CDSC (1984)
8m	Cow B278	Milk	May 84	Leeds VIC/PHLS CDSC (1984)
8n	Cow Y246	Milk	May 84	Leeds VIC/PHLS CDSC (1984)
8o	Bulk milk	Milk	May 84	Leeds VIC/PHLS CDSC (1984)
8p	Horse	Vagina	May 84	Leeds VIC/PHLS CDSC (1984)
New Mexico outbreak				
9a–h	Patients	Blood	Jul–Sept. 83	Dr Facklam/MMWR (1983)
9i	Food	Cheese	Jul–Sept. 83	Dr Facklam/MMWR (1983)
9j	Food	Milk	Jul–Sept. 83	Dr Facklam/MMWR (1983)
Northallerton (Yorkshire) outbreak				
10a	Patient	Sore throat	Apr 82	—/Barnham *et al.* (1983)
10b	Patient	Sore throat	Apr 82	—/Barnham *et al.* (1983)
10c	Patient	Sore throat	Apr 82	—/Barnham *et al.* (1983)
10d	Patient	Throat	July 82	—/Barnham *et al.* (1983)
Romania nephritis outbreak				
11a	Patient (L.V.)	Sore throat	1968	U of M 73–112/Duca *et al.* (1969)
11b	Outbreak collection, carrier		1968	Prof Duca/Duca *et al.* (1969)
Veterinary infections				
12	Piglet	Necropsy	Nov. 84	Thirsk VIC (farm, Darlington)/—
13a	Cow	Udder (mastitis)	Feb. 85	Thirsk VIC (farm, Ravenscar)—
13b	Same cow	Udder (mastitis)	Mar. 85	Thirsk VIC (farm, Ravenscar/—
13c	Mare A	Vagina	Mar. 85	Thirsk VIC (farm, Ravenscar)/—
13d	Mare B	Vagina	Mar. 85	Thirsk VIC (farm, Ravenscar)/—

C.s.f, cerebrospinal fluid; VIC, Ministry of Agriculture Fisheries and Food, Veterinary Investigation Centre; PHLS CDSC, Public Health Laboratory Service, Communicable Disease Surveillance Centre; MMWR, Morbidity and Mortality Weekly Report, Center for Disease Control, Atlanta; U of M, University of Minnesota streptococcal collection no.; Thirsk is in North Yorkshire.

serum (prepared at the Streptococcus Reference Unit, CPHL, Colindale) in a double diffusion precipitation test in agarose gel (Lancaster & Sherris, 1960).

The biochemical methods used were the same as those employed by Colman & Ball (1984). The API 20 STREP kit was used (API Laboratory Products Ltd., Basingstoke, Hants), supplemented with tests for resistance to optochin (5 μg disk) and bacitracin (0·1 unit disk) and also for production of an extracellular polysaccharide in a sucrose medium (TYC agar, Lab M, Salford, Lancs).

Surface T-antigen typing. All strains were screened in the Colindale laboratory for the presence of T-protein antigens according to the scheme developed by Efstratiou (1983) for typing human strains of group C and G streptococci.

Bacteriocin typing. Inhibitor 'fingerprinting' was performed in the Dunedin laboratory essentially as described by Tagg & Bannister (1979). The test medium was Columbia agar base (Gibco Laboratories) containing 5% (v/v) human blood and poured on a base layer of saline agar. For producer (P)-typing the test strain was grown as a diametric streak culture at 32 °C for 18 h before removing the growth, sterilizing the surface by exposure to chloroform vapours and then cross-inoculating the nine standard indicator cultures.

Six standard producers (P1–P6) were used for sensitivity (S)-typing. Producers P1–P5 were grown as streak cultures for 24 h at 32 °C. In this study P1 was incubated anaerobically, since this has been found (Tagg & Bannister, 1979) to significantly enhance its inhibitory activity and overcomes some problems of variable production from run to run. P6 was incubated at 37 °C in 5% CO_2 in air. The test strains were cross-inoculated after scraping and chloroforming the producer streaks.

The P-type and S-type results of the test strains represent in code form the patterns of inhibition of the nine indicators and sensitivity to the six producers respectively.

Bacteriophage typing. A group-C bacteriophage typing system was specially developed for this study in the Minneapolis laboratory. Mitomycin C induced lysate from 72 group-C cultures representing 16 areas of the world were examined for the presence of phage in the form of plaques or lysis on 12 group-C indicator strains. Thirty of the cultures produced lysis or plaques. Twelve of these lysogenic strains were then selected for phage typing on the basis of their lysates yielding phage plaques upon dilution and demonstrating a unique pattern of lysis. Two virulent bacteriophages were propagated by infection on indicator strains and added to the panel to make a total of 14 phages.

Bacterial cultures were grown in 549 broth which consisted of 8% Proteose Peptone No. 3 (Difco Laboratories, Detroit MI 48232), 0·2% Yeast Extract (Difco), 40 mM Hepes buffer (U.S. Biochemical Corp., Cleveland OH 44128) and adjusted to pH 7·7 with 5 N-NaOH. After autoclaving the broth was completed by the addition, to a final concentration, of 14 mM glucose and 2·7 mM-$CaCl_2$.

Lawns for the detection of phage plaques were grown on 749Y plates consisting of 4% Proteose Peptone No. 2 (Difco) 80 mM Hepes buffer, 130 mM-NaCl, adjusted to pH 6·9 with 5 N-NaOH and 1% Noble Agar (Difco). The agar medium after autoclaving was completed with the addition of 6 mM glucose, 1·8 mM-$CaCl_2$, 1 mM-$MgSO_4$, 5% horse serum and 37 units/ml hyaluronidase (Sigma).

The method for phage typing group-C streptococci was essentially as described

Table 2. *Biochemical identification of* S. zooepidemicus *isolates*

Isolate no.	VP	HIP	ESC	PYR	AGL	GUR	BGL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLY	BHS	API profile no.
Sporadic infections																						
1	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	+	+	+	4463607
2	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	+	+	+	4463607
3	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	+	−	−	+	+	+	4463617
4	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	+	+	+	4463607
5	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	+	+	+	4463607
6a–c	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	+	+	+	4463607
7	−	−	+	−	−	+	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+	4461617
Halifax outbreak																						
8a–o	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	+	+	+	4463607
8p	−	−	+	−	−	+	−	+	+	+	−	−	−	+	+	−	−	−	+	+	+	4461607
New Mexico outbreak																						
9a–j	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	+	+	+	4463607
Northallerton outbreak																						
10a–c	−	−	−	−	−	+	+	+	+	+	−	−	−	+	+	−	−	−	+	+	+	0471607
10d	−	−	+	−	−	+	−	+	+	+	−	−	−	+	+	−	−	−	+	+	+	4461607
Romania outbreak																						
11a	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	+	+	+	4463607
11b	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	+	+	+	+	4463647
Veterinary infections																						
12	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	+	+	+	4463607
13a–d	−	−	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	+	+	+	4463607

VP, Acetoin production.
HIP, Hippurate hydrolysis.
ESC, Aesculin hydrolysis.
PYR, Pyrrolidonylarylamidase.
AGL, Alpha galactosidase.
GUR, Beta glucuronidase.
BGL, Beta galactosidase.
PAL, Alkaline phosphatase.
LAP, Leucine aminopeptidase.
ADH, Arginine hydrolysis.
RIB, Fermentation of ribose.
ARA, Fermentation of L arabinose.
MAN, Fermentation of mannitol.
SOR, Fermentation of sorbitol.
LAC, Fermentation of lactose.
TRE, Fermentation of trehalose.
INU, Fermentation of inulin.
RAF, Fermentation of raffinose.
AMD, Fermentation of starch.
GLY, Fermentation of glycogen.
S, Beta haemolysis on Columbia agar (Oxoid CM 331) with 5% horse blood.

by Skjold & Wannamaker (1976) and Skjold *et al.* (1983) for group-A M49 streptococci, with a few modifications. The streptococcal lawns were made on 749Y plates with a 1 in 5 dilution of culture prepared by making two consecutive 1% 18 h transfers in 549 broth at 35 and 26 °C respectively. *S. zooepidemicus* was phage-typed with 14 bacteriophages by applying two dilutions of phage on lawns at RTD (near confluent lysis) and at 10 × RTD (confluent lysis). Lawns which demonstrated 50 or more plaques at either dilution of phage were considered positive.

Minimum inhibitory concentrations (MIC) of three antibiotics. MIC's of penicillin, tetracycline and erythromycin were determined by inoculation of the isolates of *S. zooepidemicus* on to Petri dishes containing Iso-sensitest Agar (Oxoid, code CM471) with 5% defibrinated horse blood, incorporating doubling dilutions of antibiotic (Mast Adatabs; Diamed Dignostics Ltd, Merseyside). Organisms were grown for 24 h in Todd Hewitt Broth (BBL 11736; Beckton Dickinson UK Ltd,

Table 3. *Results of bacteriocin typing of* S. zooepidemicus

Isolate no.	P-type	S-type
Sporadic infections		
1	000	57
2	000	57
3	000	57
4	000	53
5	000	53
6a–c	000	53
7	004	57
Halifax outbreak		
8a–p	266	57
New Mexico outbreak		
9a–f, i, j	000	53
9g, h	226	52
Northallerton outbreak		
10a	000	53
10b–d	000	57
Romania outbreak		
11a	407	53
11b	000	57
Veterinary infections		
12	000	53
13a–d	000	53

Oxford), cultures were well shaken, diluted 1 in 50 in sterile saline solution and dispensed to the dishes in 10 μl amounts using a multipoint inoculator (Mast Scan 100; Diamed). The dishes were incubated for 24 h in air and MIC's read and recorded as the lowest antibiotic concentrations completely inhibiting growth.

RESULTS

Colonial morphology

At 24 h, colonies varied between 0·5 and 1·5 mm diameter, were typically opaque and circular, with an entire edge, convex elevation and smooth surface. A few strains showed umbonate colonies. Isolate no. 1 gave a mixed appearance with some very mucoid colonies spreading along the lines of inoculation. The colonies of all isolates were surrounded by wide zones of beta-haemolysis.

Identification/biotype

Extracts of each isolate gave positive results with the group-C reagent in agglutination tests and reactions of identity with stock extracts of Lancefield group C.

Results of the biochemical identification tests are given in Table 2. Reactions that were generally given by the species included the fermentation of sorbitol, lactose, starch and glycogen, production of beta-glucuronidase and phosphatase

Table 4. *Patterns of bacteriophage susceptibility of* S. zooepidemicus

Isolate no.	Susceptibility to phage no.													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sporadic infections														
1	.	+	.	+	.	+	.	.	.	+	.	+	.	+
2	+	.	.	.	.	.	.	.	.	.	.	+	.	.
3	.	.	.	.	.	.	.	.	.	.	.	+	.	.
4	.	.	.	.	.	.	.	.	.	.	.	+	.	.
5	.	+	+	+	.	+	.	+	.	+	+	+	+	+
6a	.	.	.	+	.	+	.	.	+	.	.	+	.	.
6b,c								No lysis						
7	.	.	.	+	.	+	.	.	.	.	.	.	.	+
Halifax outbreak														
8a–d, g, l, p	.	+	.	.	.	.	+	.	.	.	.	+	.	.
8e, f, i–k, o	.	+	.	.	.	.	+	.	.	.	+	+	.	.
8h	.	+	.	.	.	.	+	.	.	.	.	.	.	.
8m,n								No lysis*						
New Mexico outbreak														
9a, b, d–f, i	.	+	+	+	.	+	.	.	.	.	.	+	.	+
9c	.	.	+	+	.	+	.	.	.	.	.	+	.	+
9j	.	+	.	.	.	.	.	.	.		.	+	.	.
9g, h	.	.	.	.	.	.	+	.	.	.	+	+	+	.
Northallerton outbreak														
10a	.	.	.	+	.	+	.	.	.	.	.	.	.	.
10b–d	.	.	.	.	.	.	.	.	.	.	.	+	.	.
Romania outbreak														
11a	.	.	.	.	.	.	.	.	.	+	.	.	.	.
11b								No lysis						
Veterinary infections														
12	.	.	.	.	.	.	.	.	.	.	.	+	.	.
13a, c	.	.	.	.	.	.	.	.	.	.	.	+	.	.
13b								No lysis						
13d	.	.	.	+	.	+	.	.	.	.	.	+	.	+

*, 8n possibly type 7/12.

and the failure to produce pyrrolidonylarylamidase, hydrolyse hippurate or give the Voges–Proskauer reaction. All but 8 of the isolates gave the API profile number 4463607; unusual reactions included a failure to ferment ribose (seen in 6 isolates), positive trehalose fermentation (2 isolates) and late raffinose fermentation (1 isolate).

All the isolates were resistant to disks containing 0·1 unit bacitracin and 5 µg optochin, and none produced dextran or levan from sucrose.

T-antigen typing

T-protein antigens were not detected on any isolate using the collection of antisera prepared with human isolates of the Lancefield groups A, C or G.

Table 5. *Minimum inhibitory concentrations (MIC) of three antibiotics against* S. zooepidemicus

Isolate no.	MIC (mg/l) of antibiotic		
	Penicillin	Tetracycline	Erythromycin
Sporadic infections			
1	0·008	1·0	0·008
2	0·008	4·0	0·03
3	0·015	16·0	0·06
4	0·008	1·0	0·06
5	0·008	2·0	0·06
6a–c	0·015	4·0	0·06
7	0·015	4·0	0·06
Halifax outbreak			
8a, b, d–h, j, k, p	0·008	4·0	0·06
8c, i	0·008	4·0	0·03
8m, n	0·008	2·0	0·06
8l	0·008	2·0	0·03
8o	0·008	0·5	0·015
New Mexico outbreak			
9c–f, j	0·015	8·0	0·06
9a, b, i	0·015	4·0	0·06
9g, h	0·015	2·0	0·06
Northallerton outbreak			
10a, b	0·015	4·0	0·06
10c, d	0·008	8·0	0·06
Romania outbreak			
11a	0·008	8·0	0·06
11b	0·008	4·0	0·06
Veterinary infections			
12	0·015	8·0	0·06
13a, b	0·008	4·0	0·03
13c	0·008	4·0	0·06
13d	0·015	4·0	0·06

Bacteriocin typing

Results of the bacteriocin typing tests are shown in Table 3. The scheme revealed 5 P-type and 3 S-type patterns in the collection of *S. zooepidemicus*. There were differences between each of the major outbreak collections (series 8, 9, 10 and 11). Isolates 9g, h were distinct in both P- and S-type from the other isolates in the New Mexico series; in the Northallerton series isolate 10a was distinct from the others in S-type.

Bacteriophage typing

Bacteriophage typing results are shown in Table 4. As with the bacteriocin typing, there were differences between each of the major outbreak collections; the scheme showed a difference between isolates 9g, h and others in the New Mexico series, and between isolate 10a and the others in the Northallerton series. Most of the isolates were susceptible to phage number 12 but none to number 5 in the panel.

Table 6. *Predominant patterns of combined bacteriocin, bacteriophage and biotyping results in the* S. zooepidemicus *collection*

Combined results of typing by bacteriocin: bacteriophage: API profile	Isolate numbers showing the pattern
P000, S53: 12: 4463607	4, 12, 13a, c
P000, S53: 2/3/4/6/12/14 complex: 4463607	9a–f, i
P226, S52: 7/11/12/13: 4463607	9g, h
P266, S57; 2/7/11/12 complex: 4463607	8a–g, i–l, o
P000, S57: 12: variable	3, 10b–d

Antibiotic MIC studies

MIC's of penicillin, tetracycline and erythromycin against the isolates of *S. zooepidemicus* are shown in Table 5. All isolates were susceptible to penicillin (MIC range 0·008–0·015 mg/l) and erythromycin (range 0·008–0·06 mg/l) but resistant to tetracycline (range 0·5–16·0 mg/l).

Combined typing patterns

Combining the results of typing by bacteriocin, bacteriophage and the API profile, isolates in the collection were grouped into five main patterns, as shown in Table 6. Other combined patterns were seen with individual isolates.

DISCUSSION

Human infection with *S. zooepidemicus* appears to be a rare event and has mostly followed close exposure to horses or the consumption of contaminated dairy products (Barnham, Thornton & Lange, 1983). When it does occur the infection may be overwhelming, as in many patients in the recent milk- and cheese-borne outbreaks (Ghoneim & Cooke, 1980; Morbidity and Mortality Weekly Report, 1983; PHLS Communicable Disease Surveillance Centre, 1984, unpublished). In view of its severity, *S. zooepidemicus* infection has been considered the most notable milk-borne disease of the last few years in Britain (Sharp, Paterson & Barrett, 1985). The infection is also of special interest as a cause of PSGN, which until recently was thought only to follow infection with *S. pyogenes* (Duca *et al.* 1969; Barnham, Thornton & Lange, 1983).

We put together an international collection of isolates from human infection in order to study and compare the organisms, and to develop a typing system that might help in epidemiological studies. Organisms from the first recorded outbreak of systemic infection, in Leeds in 1979 (Ghoneim & Cooke, 1980), were unfortunately not saved but we have assembled isolates from all the other recorded outbreaks and from a range of sporadic infections. Yorkshire has been a good area to gather the organisms as the practice of drinking raw milk is particularly common here (Sharp, Paterson & Barrett, 1985).

We found that the API 20 STREP profile led to the identification of the organisms but was less helpful as a biotyping tool by itself, as most of the strains gave the same profile, 4463607. Isolates from patients and cowman in the Northallerton outbreak were indistinguishable by bacteriocin and bacteriophage

typing although there were differences in two biochemical reactions between them (profiles 0471607 and 4461607 respectively).

A serotyping scheme for isolates of *S. zooepidemicus* from horses was reported by Bryans & Moore (1972), but this work was discontinued and the sera are no longer available (personal communication). They were able to detect a series of 15 type-specific protein antigens, acid extracts of which were labile to trypsin or pepsin. Mihalcu *et al.* (1982) raised three antisera which typed 42% of a collection of strains of Lancefield group C *S. equisimilis* but they completely failed to type *S. zooepidemicus*. We applied the T-protein antigen serotyping scheme developed by Efstratiou (1983) for human isolates of Lancefield group C and G streptococci but obtained no positive results. The system achieves 76% typability with human strains of *S. equisimilis* and the failure to type any of the strains of *S. zooepidemicus* emphasizes the antigenic differences between these species.

The only previous study of bacteriocin typing of group-C streptococci was that of Schofield & Tagg (1983), who found that certain strains of *S. zooepidemicus*, *S. equisimilis* and *S. dysgalactiae* produced bacteriocin-like inhibitors. Four of 8 strains of *S. zooepidemicus* produced inhibitors, all giving different P-types.

In the present study a new P-type (266), not seen with any previously tested streptococcus, was found in the organisms from the Halifax outbreak (series 8). Isolates 9g, h in the New Mexico collection gave a P-type (226) identical to the strain 4881 in the earlier study of Schofield & Tagg (1983), an isolate from an aborted foal in New Zealand. S-typing gives some useful further discrimination, particularly amongst the non-producer strains.

The group-C phage-typing system described here was developed specially for the purpose and modelled on the group-A type-49 phage-typing system of Skjold & Wannamaker (1976). The system appears to give useful discrimination, with differences shown between each of the main outbreak collections. Within outbreaks the differences were minor, as in series 8, or major, as in series 9 (where isolates 9g, h appeared quite different) and 10 (where 10a was different); these results concurred with the findings of bacteriocin typing and DNA fingerprinting (see Skjold *et al.* 1987), suggesting that in both the New Mexico and Northallerton outbreaks more than one strain was involved. Additional phage may be needed in the typing panel to differentiate the strains which are susceptible only to phage number 12. Human and animal strains of *S. zooepidemicus* were not susceptible to phage in the lysotyping scheme developed for group C streptococci by Mihalcu *et al.* (1982).

Many strains that show the common API profile number 4463607 can be distinguished by the combination of bacteriocin and bacteriophage typing, as shown in Table 6. We do not know if these groups could be divided further by elaboration of the typing systems, but they already give helpful information about the collected strains. Acute PSGN followed infection with isolates 5, 10a, c and 11a and these have come out differently in our combined typing system. Indistinguishable strains were found within many of the epidemiological clusters: the cow and mare A at Ravenscar (series 13), the patients, cow and milk at Halifax (series 8; the equine strain here varied only in the failure to ferment ribose), and many patients, the cheese and the milk from New Mexico (series 9). DNA fingerprinting of the organisms (Skjold *et al.* 1987) confirms many of these

distinctions and further discriminates between sporadic isolates of the same combined bacteriocin-, bacteriophage- and bio-type.

Carriage and infection with *S. zooepidemicus* is especially common in horses (Stableforth, 1959; Bryans & Moore, 1972; Erickson, 1980) and this might be a source of infection on a farm, perhaps by common grazing, environmental contamination or via handlers, to produce the unusual bovine mastitis that is such a hazard to man. The similar typing patterns of the equine and bovine isolates in the mastitis episodes (series 8 and 13) lend some support to this concept.

To gain more information on the nature and circumstances of *S. zooepidemicus* infection we suggest that isolates of group C streptococci from man should be identified to species level, at least in the following situations: when infection is severe, invasive or followed by PSGN, when there seems to be a link with animals, or when clusters occur. Examination of human and associated animal isolates by a typing scheme such as that described here, perhaps augmented with DNA fingerprinting, should then help to clarify the patterns of infection.

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The serotyping of hospital strains of streptococci belonging to Lancefield group C and group G

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SUMMARY

A collection of more than 300 strains belonging to either Lancefield group C or group G was made. The cultures had been isolated either sporadically from patients with serious disease or as apparent clusters from various nosocomial outbreaks. T-protein antigens were sought. So far, nine distinct serotypes have been found among the group G streptococci and seven serotypes amongst the *Streptococcus equisimilis* (group C) strains. Of the sixteen serotypes, four were the original T-types 7, 16, 20 and 21 described by Griffith (1934). Because of the similarities of representatives of the two groups to Lancefield group A streptococci (Griffith, 1934; Maxted & Potter, 1967) a few strains not unexpectedly carried T antigens usually seen in group A streptococci. Using this scheme it has been possible to serotype 76% of *S. equisimilis* strains of human origin and 82% of group G streptococci from human clinical material. A small collection of group C and group G streptococci of animal origin could not be serotyped with the experimental T-antisera.

INTRODUCTION

The haemolytic streptococci belonging to Lancefield group C and group G can be isolated as commensals from the throat, from cultures of the umbilical stumps of neonates and occasionally from routine vaginal cultures. They are, however, also able to cause serious disease in man such as pneumonia, cellulitis or endocarditis (Mohr *et al.* 1979; Ancona, Thompson & Ferrieri, 1979). Asymptomatic existence in the upper respiratory tract may be followed by complications such as acute pharyngitis and lymphadenitis (Hill *et al.* 1969). In animal populations groups C and G streptococci have been shown to cause serious infections (McFadden & Boon, 1949; Biberstein, Brown & Smith, 1980).

There has been a steady increase in the numbers of group C and group G streptococci received in this laboratory for further examination. These include strains from systemic diseases. There have also been clusters of strains from what appear to be hospital outbreaks.

A provisional typing scheme has therefore been developed to test whether or not these organisms are epidemiologically related and also, if there is an association between a particular systemic disease and the serotype of the strain responsible. Several species of streptococci comprise each of the serological groups C and G.

One species in each group, namely *Streptococcus equisimilis* in group C and the 'large-colony' variety or *S. canis* in group G have many similarities to the major human pathogen *S. pyogenes*, Lancefield group A. Cell-wall protein antigens, notably the T proteins and M proteins, have been described in all three species (Griffith, 1934; Lawal *et al.* 1982; Maxted & Potter, 1967).

The T-protein antigen, although not a virulence factor, is an important epidemiological marker in the serological classification of group A streptococci. Griffith's (1934) original classification of pyogenic streptococci into different T-types included types 7, 16, 20 and 21 which were eventually found not to belong to Lancefield group A but to be strains of either group C or group G. Simmons & Keogh (1940) subdivided these organisms with a scheme based on a combination of serological and biochemical characteristics but their strains are apparently lost with the exception of one minute colony type strain.

The M-protein antigens of strains of group G isolated in Nigeria have been studied by Lawal *et al.* (1982). The T-protein antigens are, however, easier to study and, as with the group A streptococci, it seemed appropriate to make the first subdivision on the basis of these antigens.

MATERIALS AND METHODS

Bacteria

The streptococci used in this study were mainly from hospitals in England, but some were received from overseas. They were either random isolates from different sources and implicated in a serious disease or they were possible causes of nosocomial outbreaks. Stock cultures of group C and group G streptococci were also examined. Totals of 17 group C strains and 12 group G strains isolated from animals were included in the study. Table 1 lists the vaccine strains used for the preparation of the experimental T-antisera.

Preparation of the antisera

T-typing sera were prepared in rabbits using trypsinized whole cell vaccines. Each vaccine strain was grown in 250 ml of Colindale Todd-Hewitt broth containing 1 % (v/v) of a 10 % (w/v) sterile solution of Difco '1/250' trypsin (McLean,

Table 1. *Vaccine strains used in the preparation of antisera*

Lancefield group C		Lancefield group G	
Strain no.	T-type	Strain no.	T-type
NCTC 4540*	7	NCTC 5969	16
NCTC 5370	20	R80/4327	PT 4327
NCTC 5371	21	R80/5007	PT 5007
R80/3722†	PT 3722‡	R80/5356	PT 5356
R80/4225	PT 4225	R80/6866	PT 6866
R80/5582	PT 5582	R80/7023	PT 7023
R81/1058	PT 1058	R80/7118	PT 7118
.	.	R81/1986	PT 1986
.	.	R81/3181	PT 3181

* NCTC = National Collection of Type Culture strains.
† R = Laboratory strains from the Streptococcus Reference Unit.
‡ PT = Provisional type number.

1953). Incubation was at 30 °C for 24 h to enhance the production of the T-antigen. The cells were harvested, washed and resuspended in 25 ml of phosphate-buffered saline (pH 7·8, 0·1 M saline, 0·2 M phosphate) containing 0·5 % trypsin and left at room temperature for 24 h. The cells were then washed six times in sterile physiological saline and finally resuspended in 17 ml of saline and 3 ml of a formalin solution (8 % (w/v) formaldehyde in saline) and left at room temperature for 4 h. The formaldehyde content of the suspension was then reduced by centrifuging the suspension and replacing half the supernatant with saline. Rabbits were immunized by intravenous injections of 1 ml of the vaccine twice weekly for at least 4 weeks.

Absorption of the antisera

The antisera were absorbed with an equal volume of packed cells of an appropriate strain (Williams, 1958). Antibodies, for example, to the group G antigens were removed by absorption, repeated when necessary, with the culture, group G R80/3430 carrying the T antigen 25. Group C, R79/3540 with the T antigen 4, was used for all group C serum absorptions. The absorption suspensions were left at room temperature for 24 h or at 37 °C for 4 h. The absorbed antisera were then titrated against their homologous trypsinized vaccine strain by slide agglutination and then, finally, against a series of suspensions of known T-types for any cross-reactivity. Thiomersal (20 mg/l) was added as a preservative.

Serological methods

Cell suspensions were prepared by trypsin extraction (V. D. Allison, unpublished) as modified by Efstratiou (1980). Agglutination tests were performed by Griffith's method. Each antiserum was used at the highest dilution which gave a strong reaction. This varied between 1 in 500 to 1 in 10000 depending upon the serum.

RESULTS

Antisera were first prepared for the T antigens 7, 16, 20 and 21 and their apparent occurrence in only strains of Lancefield groups C and G was confirmed. But these sera agglutinated only about one-fifth of the strains tested. Rabbits were then immunized with strains that did not carry these antigens and a provisional set of 16 experimental sera was obtained. The vaccine strains selected were random isolates from cases of disease and from probable outbreaks of infections.

Overall, of the human isolates examined, 76 % of group C streptococci (*S. equisimilis*) and 82 % of group G could be serotyped (Fig. 1). There were 42 instances of cross-over between T antigens belonging to the two Lancefield groups. Certain strains carried T antigens belonging to the other group. This of course was also seen with the 24 strains of Lancefield groups C or G sharing the T antigens, 2, 4, 28 or 8/25/Imp 19 with strains of group A.

Group C streptococci (S. equisimilis)

As shown in Fig. 1 and from the data in Table 2 it was possible to subdivide provisionally the Lancefield group C (*S. equisimilis*) strains into 11 different serotypes. Some 24 % were, however, not typable. Many of the *S. equisimilis* strains among the random isolates (Table 2) were recovered from blood cultures from

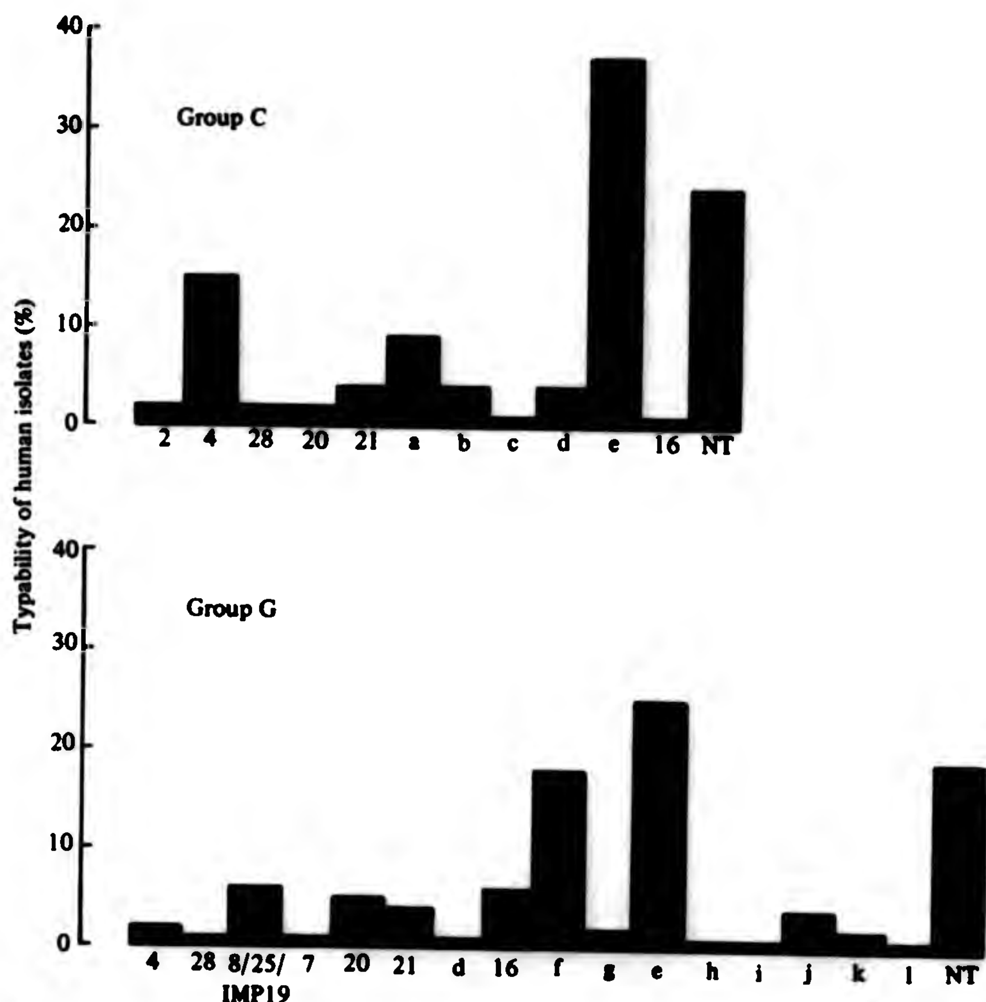

Fig. 1. Serological subdivision of Lancefield group C (*S. equisimilis*) and Lancefield group G by T-typing. NT = not typable, a = PT1058, b = PT3722, c = PT4225, d = PT5582, e = PT5007, f = 7/PT5356, g = PT4327, h = PT6866, i = PT7023, j = PT1986, k = PT3181, l = 20/PT3181.

cases of endocarditis or from throat swabs of patients with pharyngitis or tonsillitis. Of the 15 strains of group C isolated from blood cultures nine belonged to the provisional type '5007'. This is overall the commonest T-type in the collection. Three outbreaks of pharyngitis, presumably due to group C streptococci, were each caused by a single serotype. The strains from a single institution were serologically homogeneous. These included episodes of tonsillitis at two schools, with T-types PT1058 and PT3722 isolated separately at each school. The third outbreak was of pharyngitis in a military camp with T-type 4 being the serotype isolated from all throat swabs. Of the 111 cultures of Lancefield group C examined, 77 belonged to the species *S. equisimilis*. Of these strains eight were isolated from animals. There were 18 isolates of *S. equisimilis* from the outbreaks. A small group of strains belonging to other species of Lancefield group C were tested with the experimental sera but were not agglutinated (Table 3). These, with the exception of seven strains, were of animal origin.

Preliminary studies using trypsinized cells of other species of group C as immunogens were less successful (Table 4). Group antibody was produced in abundance. After absorption procedures were used to remove group antibody, slide

Table 2. *Serotype distribution of single human isolates of* S. equisimilis *Lancefield group C*

Source	No. of isolates	Serotypes											
		2*	4*	28*	20	21	PT 1058	PT 3722	PT 4225	PT 5582	16	PT 5007	NT
Skin	6	.	.	.	.	.	.	.	.	.	.	.	6
Blisters	3	.	.	.	.	.	.	.	.	1	.	1	1
Wounds	4	.	.	.	.	.	.	.	1	1	.	1	1
Leg ulcers	6	.	.	.	.	.	.	.	.	.	.	4	2
Blood	15	1	.	1	.	.	.	1	.	.	.	9	3
Brain	1	.	.	.	.	.	.	.	.	.	.	1	.
Aspirates	3	.	.	.	.	.	.	.	.	.	.	1	2
Burns	1	.	.	.	.	.	.	.	.	.	.	.	1
HVS	2	.	.	1	.	.	.	.	.	.	.	.	1
Throat	17	.	1	.	1	4	3	.	.	2	1	3	2
CSF	1	.	.	.	.	.	.	.	.	.	.	.	1
Sputum	1	.	.	.	.	.	.	.	.	.	.	1	.
Urine	1	.	.	.	.	.	.	.	.	.	.	1	.
Ear	1	.	.	.	.	.	.	.	.	.	.	1	.
No information	7	.	3	.	.	.	.	.	.	.	.	4	.
Total	69	1	4	2	1	4	3	1	1	4	1	27	20

* T antigens characteristic of some strains of *S. pyogenes* (Lancefield group A).

Table 3. *Failure to detect T antigens amongst species of Lancefield group C streptococci other than* S. equisimilis *isolated from man*

Species	No. isolated	T-type
S. dysgalactiae	5	.
S. equi	2	.
*S. zooepidemicus**	5	.
S. equisimilis†	8	.
S. milleri‡	4	.

* These include three cultures from a family living on a farm.
† All isolates from animals.
‡ Isolates from humans of *S. milleri* with the Lancefield group C polysaccharide antigens.

Table 4. *Antibody response in rabbits to trypsinized immunogens of Lancefield group C streptococci*

Species	Vaccine strains	Group antibody	T antibody
S. equisimilis	See Table 1	+	+
S. dysgalactiae	NCTC 4669	+	−
	NCTC 4335	+	−
	NCTC 4671	+	−
S. equi	NCTC 9682	+	−
S. zooepidemicus	NCTC 6179	+	−

agglutination tests with even the immunizing strains were all negative. The rabbits were then re-immunized and similar negative results obtained. This suggests that T-protein antigens may be absent from the other species of Lancefield group C.

Group G streptococci

Of the typable strains of group G streptococci, some 53% of the isolates were from hospital outbreaks. Cross-infection was suspected from the first and made more likely by the results of T-typing (see below). The data brought together in Table 5 show that the group G streptococci examined from single random isolates can be subdivided into at least fifteen T-types. Again, as with the group C streptococci, there were some instances of cross-over between particular T antigens of Lancefield groups A, C and G. These were 2, 4, 8, 25, 28, Imp 19, the types proposed here namely PT5007, PT5582 and Griffith's original types 7, 16, 20 and 21. The majority of group G strains were isolated from cases of severe diseases such as septicaemia, endocarditis, cellulitis and septic arthritis. Cultures were also recovered from patients with tonsillitis. Type associations between serious diseases and serotypes suggested that, in general, three T-types predominated among strains isolated from cases of septicaemia and endocarditis. Four-fifths of these 25 strains could be T-typed. A total of nine strains were agglutinated by both the type 7 and PT5356 sera, four by the PT5007 serum and three by the PT5582 serum. The largest groups of single isolates of group G examined were from blood, skin, leg ulcers and throat. Altogether, 73% of the random isolates were typable. This rate was increased to 82% by the inclusion of strains isolated from apparent outbreaks (Fig. 1).

Group G streptococci have been isolated recently from clusters of infections in several hospitals within the UK. They have, for instance been isolated from burns and plastic surgery units. From hospital A, many T-types were isolated. These are listed in Table 6. In the burns unit of this hospital, patients in two wards were involved in apparent outbreaks of group G infections. There appeared to be evidence of nosocomial spread in ward 1 with five isolations of T4 strains. The possibility of cross-infection in ward 2 was also considered. Strains from maternity units were also isolated. In one instance, cultures were isolated from a woman with a post-partum infection and representatives of the same type (7/PT5356) were isolated from a sample of bath water taken from the bath used by this patient. Two pairs of mother and baby related strains were seen. One pair of cultures was T-type 20 and the other 7/PT5356. An outbreak in an operating theatre of group G infections occurred at another hospital with serotype 16 isolated from all sources including environmental samples from the floor, floor mats and the footwear of staff. A total of 23 strains were isolated from leg ulcers but only 12 could be typed with the existing set of antisera.

As with group C, those group G strains isolated from animals could not be typed.

DISCUSSION

Comparatively little work has been done on the typing of human isolates of streptococci of Lancefield group C and group G, since the original work of Griffith, almost fifty years ago. This study is hopefully an extension of Griffith's original

Table 5. *Serotype distribution of non-related human isolates of Lancefield group G streptococci*

Source	No. of isolates	Serotypes: 28	8/25/ Imp 19	7	20	21	PT 5582	16	7/PT 5356	PT 4327	PT 5007	PT 6866	PT 7023	PT 3181	20/PT 3181	NT*
Skin	20	.	2	1	1	1	.	.	5	1	4	.	.	.	.	5
Blisters	3	.	.	.	.	.	.	1	.	.	2	.	.	.	.	0
Wounds	10	.	.	.	2	1	.	1	2	.	.	.	.	.	.	4
Leg ulcers	23	2	5	.	.	.	.	1	1	.	3	.	.	.	.	11
Blood	25	.	1	.	.	1	3	.	9	.	4	.	.	1	1	5
Biopsy site	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	0
Aspirates	5	.	2	.	.	.	.	1	1	.	.	.	.	.	.	1
Ear	4	.	.	.	1	.	.	.	1	1	.	.	.	.	.	1
Nose	3	.	.	.	.	.	.	.	1	.	2	.	.	.	.	0
Throat	20	1	.	.	1	.	.	.	3	2	4	.	1	1	.	7
Abscesses	5	.	.	.	1	.	.	.	1	.	1	.	.	.	2	0
Burns	1	.	.	.	.	.	.	.	.	.	1	.	.	.	.	0
Urine	1	.	.	.	.	.	.	.	.	.	1	.	.	.	.	0
Vagina	2	.	.	.	.	.	.	.	1	.	.	.	.	.	.	1
Umbilicus	1	.	.	.	.	.	.	.	1	.	.	.	.	.	.	0
No information	7	1	1	.	1	.	.	2	1	.	.	1	.	.	.	0
Total	131	4	12	1	7	3	3	6	27	4	22	1	1	2	3	35

* Not typable.

Table 6. *Origin and serotype distribution of clusters of Lancefield group G streptococci from apparent outbreaks*

Apparent outbreaks	Source	No. of isolates	T-type
Eye infections in one ward	Eyes	4	PT 5007
Self infection	Eye, finger	2	16
Operating theatres	Nose or throat of staff plus environmental isolates	6	16
School A	Nose, throat	5	3; type 21 2; PT 4327
Hospital A			
Burns Unit:			
Ward 1	Wounds	5	4
Ward 2	Wounds	19	5; 7/PT 5356 1; PT 5007 1; type 4 10; PT 1986 2; NT
Maternity Unit:			
Patients 1–4	Wounds	4	20; 21; PT 5007 & type 28
Patient 5	Perineum, bath water, bath water swab, toilet seat	4	7/PT 5356
Hospital B			
Ward 1	Wounds	1	21
Ward 2	Wounds	1	7/PT 5356
Ward 3	Wounds	2	PT 3181
Ward 4	Throat, nose, hairline	7	2; 7/PT 5356 5; PT 5007
Hospital C			
Two patients in one ward	Ulcers, wounds	2	7/PT 5356
Hospital D			
Special care baby unit	Infected umbilicus, septic spots	2	PT 5007
Hospital E			
Maternity Unit:			
mother; baby	HVS; nose	2	7/PT 5356
patient	HVS, urine	2	7/PT 5356
Hospital F			
Maternity Unit			
mother; baby	HVS; rectal swab, gastric aspirate and ear	4	20
Hospital G			
Patients in one ward	Impetigo, leg ulcer	2	NT
Hospital H*			
Burns Unit	Raw areas & environment	44	26; PT 5007 18; six other types

* Survey of group G streptococcal infection in a burns unit.

work and the system developed should be useful for serotyping clinical isolates, either random strains or more particularly as clusters.

Experience with T-typing of group A streptococci has shown that this method, used alone, will effectively indicate that an outbreak is occurring. For example, in the United Kingdom, the strains recovered from an outbreak of scarlet fever

in a school and carrying the M-type 3 antigen will also have the T-type 3 antigen. For this reason some reference laboratories use T-typing alone for the subdivision of group A streptococci. But, for further study of these strains, as with Lancefield group A, examination for possible M antigens could be the definitive procedure.

The T-protein antigens of Lancefield group C and group G streptococci were found to be excellent immunogens. The titres of agglutinating sera ranged from 1 in 500 to 1 in 10000. It is unusual to find such high titres when group A strains are used as vaccines. In parallel with this there were also unfortunately high titres of group-specific antibody. Heavy absorption procedures were required. Taken together these results suggest that future experiments should explore the use of purified T proteins as immunogens and this is being done.

Many strains were isolated from blood cultures with the provisional types 5007 and the complex 7/5356 being the main serotypes for both Lancefield group C and group G. Further absorption of the sera could perhaps eliminate these cross-reactions between strains belonging to the different groups, but it does not appear necessary at present for epidemiological studies. It is noteworthy, however, that the T-type 7 antigen occurred alone in group C strains and in association with the PT5356 antigen in group G strains bar one.

It is suggested that further work on the preparation and perhaps purification of anti-T sera would extend this provisional serotyping scheme to strains presently untypable. That this could be worthwhile is indicated by the many strains isolated from leg ulcers that could not be typed. As far as is known the T antigens of the group A streptococci are not directly related to the virulence of these organisms and there is as yet no evidence to suggest whether or not the T antigens of the streptococci of group C or group G inhibit phagocytosis. By analogy, this would seem unlikely. Any exploration of mechanisms of pathogenicity would probably need to be preceded by an examination of these organisms for M antigens and preliminary work along this line has begun.

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Table 1 The yearly changes of T-types of group A streptococci isolated from throats of five cases during five years (1977-1982)

	date of throat culture	T-types of Group A streptococci				
		H.T.	E.A.	M.F.	M.I.	A.O.
1977	Sep. 8		1	1	12	12
	Oct. 18	12(125)	1(50)	1(166)	4(333)	12(125)
1978	Jan. 11	1	1	1	4	12
	Feb. 14	1	1	1	4	12
	Oct. 17	1	1	1		12
	Nov.10	1(1250)	1(125)	1(166)	1(333)	12(125)
1979	Jan. 18	1	1	13	1	12
	Feb. 16	1	1	13	1	12
	Sep. 5	1	1	13	1	12
	Oct. 4	1(500)	1(166)	13(166)	1(250)	12(125)
1980	Jan. 11			22		12
	Feb. 14			22	1	
	Sep. 9		18	23		B*
	Oct. 16	1(166)	18(125)	22(166)	(125)	12(12)
1981	Jan. 13	1	18	22		12
	Feb. 6	1	18	22		12
	Sep. 30	1	6		6	6
	Oct. 29	33(333)	18(125)	13(333)	6(833)	6(625)
1982	Jan. 14	28	6	6	6	6
	Feb. 9	13	6	9	6	6

*B: Group B streptococci   ( ): ASO titer (Todd unit)

streptococci, serial isolations of the same type of strains of group A streptococci were seen for rather long duration (Table 1).

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The investigation of outbreaks of infection caused by human strains of Lancefield group C or group G streptococci

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Pyogenic streptococci of the Lancefield groups C or G have been isolated from outbreaks of human infection[1,2]. A typing scheme would be helpful for the investigation of similar incidents and methods have been described that subdivide these organisms using bacteriophages[3], bacteriocines[4] or M-protein antigens[5]. T-protein antigens have also been detected on these streptococci[6,7] and were used to construct a provisional typing scheme[8] the application of which is described here.

Serological methods. Initially, standard methods employing whole cell vaccines were used for the production of agglutinating sera. The need to absorb the typing sera was however largely avoided by extracting the T antigens[9] and using the extracts as immunogens. The most useful types, 18 so far, are listed in Table 1.

Table 1 Strains used in the preparation of antisera

Lancefield group	T-type	Strain number
C	7	NCTC 4540
C	20	NCTC 5370
C	21	NCTC 5371
C	200	NCTC 11552
C	201	NCTC 11553
C	202	NCTC 11564
C	203	NCTC 11565
C	204	NCTC 11629
G	16	NCTC 5969
G	300	NCTC 11554
G	301	NCTC 11555
G	302	NCTC 11556
G	303	NCTC 11557
G	304	NCTC 11566
G	305	NCTC 11567
G	306	NCTC 11568
G	307	NCTC 11569
G	308	NCTC 11630

T antigens. The extracted T antigens were contaminated with trypsin and the two could not be separated by gel chromatography. The enzyme was removed by affinity chromatography using rabbit antibodies to trypsin coupled to cyanogen-bromide activated Sepharose 4B. The proteins from types 301 and 308 were found to be acidic proteins with pI's about 4-5 and molecular weights, estimated by polyacrylamide gel electrophoresis, of approximately 28000 Daltons.

Serotyping scheme. Strains to be tested are grown in Todd Hewitt broth supplemented with trypsin and representatives of group C are tested first with the pooled sera numbered 1, 2, 4 and 5 and those of group G

Table 2 Serotyping pools

Pool 1	Pool 2	Pool 3	Pool 4	Pool 5
7	16	303	200	201
21	20	305	202	204
301	300	306	203	
302	304	308		
	307			

with the pools numbered 1, 2 and 3 (Table 2). Individual sera are then used. If none of the five pools of sera causes agglutination the bacterial cell suspension is tested against typing sera numbered 2, 4, 28 and 8, 25 and Imp 19 that have been prepared for typing group A streptococci. As when T-typing group A streptococci multiple reactions are occasionally seen: the most common being with serotypes 7 and 302 or 301 and 305.

Type numbers. The type numbers 7, 16, 20 and 21 were used by Griffith for streptococci of the groups C and G[6]. It is proposed that new T-types associated with group C be given numbers in a series beginning with 200 and that numbers beginning at 300 be used for the group G strains.

Typing of random isolates. The percentage typability of a thousand single isolates was 80% for strains of both Lancefield groups. There were no clear-cut associations linking the occurrence of particular serotypes with sources or disease states but Griffiths original T-types (7, 16, 20 and 21) were more common in throat swabs than from other sources.

Outbreaks caused by group C streptococci. The largest single outbreak studied occurred during the summer of 1983 and was of sore throat affecting, predominantly, the female staff of a district hospital. The most likely source was a salad containing sliced hard-boiled eggs. All 137 cultures belonged to the serotype 204 and were isolated from throat swabs. None was isolated from nose swabs. The subjects responded promptly to antibiotic treatment.

Streptococcus zooepidemicus caused two outbreaks of infection with some deaths and in each the consumption of unpasteurised milk was the probable source. Attempts to demonstrate T antigens in representatives of this species failed.

Outbreaks caused by groups G streptococci. Strains from several outbreaks of nosocomial infection were available. In one outbreak in a maternity unit perineal wounds on 12 mothers became infected. Group G streptococci of type 308 were isolated from all 12. Representatives of type 7/302 were isolated from two further mothers. None of the streptococci isolated from nose or throat swabs taken from members of staff could be typed. Cultures of type 308 were isolated from environmental swabs taken in the bathroom used by the patients.

Streptococci of Lancefield group G are often isolated from swabs taken routinely from burns patients. During a period of one year in one unit a total of 55 isolations were made of which 28 belonged to type 301. Among the type 301, 20 strains were recovered from patients, five were from settle plates and three were from throat swabs taken from members of staff. Six strains belonged to type 16, five carried antigens of the 8/25/Imp 19 complex, four were agglutinated by 7/302 sera, two by type 300 sera, one by type 21 and nine could not be typed. The recovery of group G streptococci from a recently grafted area was associated with necrosis of the skin graft at that site.

Food-borne outbreak of sore throat caused by group G streptococci has been reported[10]. All ten strains examined belonged to type 301.

Asymptomatic carriage of group G streptococci. A survey was made of nose and throat carriage of pyogenic streptococci in two residential institutions. The carriage rate for group G streptococci approached 10% (Table 3). In the first establishment ten of the 17 strains isolated belonged to serotype 300 and in the second, nine of the 20 strains were identified as type 308.

Table 3 Isolation of streptococci from throat swabs

	Sample	Group A	C	G
Institution A	182	5	14	17
Institution B	220	19	7	20

In each institution a total of seven different serotypes were recovered. The simplest explanation of these findings is that, as with group A streptococci, strains of Lancefield group G can be passed from person to person without necessarily causing disease.

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