THE ANTIGENIC STRUCTURE AND VIRULENCE OF NEISSERIA GONORRHOEAE

by

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ABSTRACT

A bactericidal assay has been developed which permits the identification of different serotypes within 'Neisseria gonorrhoeae'. Neutralisation experiments have shown that the antigens concerned are lipopolysaccharides. The antibodies in a normal human serum which, with complement, were bactericidal for 20 of the 60 strains tested were shown to cross react with organisms in the normal flora. Normal human sera varied considerably in their ability to kill subcultured gonococci.

A haemagglutination test was developed which detected antibodies to gonococcal lipopolysaccharide in approximately 90% of patients with acute gonorrhoea and less than a quarter of normal controls. Using this test type specific immunodeterminants were detected on the lipopolysaccharide.

In the host gonococci can persist even though immunoglobulins G, M and beta - 1 C/beta - 1 A could be detected in the inflammatory exudate by immunodiffusion. Host grown gonococci were found to be resistant to killing by the homologous patients' sera while the same organism after minimal subculture was sensitive. It is postulated that gonococci in the host possess a protective factor overlaying the vulnerable lipopolysaccharide. The rapid loss both of this factor and of gonococcal virulence on subculture suggests that, for the first time, a parameter is available for assessing gonococcal virulence in the laboratory.

The virulence of gonococci was not found to be related to their colonial type or their ability to grow in the 8 day embryonated egg.

Although gonococci proved to be avirulent for mice, when isolated from the host defence systems in a diffusion chamber within the peritoneal cavity the organisms multiplied but remained sensitive to serum killing. To overcome the problem of maintaining virulent gonococci a method was developed of preserving host grown gonococci in liquid nitrogen with negligible loss of viability.

CONTENTS

		Page No.
	Acknowledgements	1
	Abbreviations	2
CHAPTER 1.	Review of the literature.	
	The gonococcus as a successful pathogen.	
1.1.1.	Historical background	3
1.1.2.	Incidence of gonorrhoea	3
1.1.3.	The clinical manifestations of gonorrhoea ...	5
	The antigenic structure of Neisseria gonorrhoeae.	
1.2.1.	Introduction	6
1.2.2.	The heterogeneity and instability of gonococcal antigens	6
1.2.3.	The chemical nature of the type specific antigens of gonococci	8
1.2.4.	The evidence for capsular antigens	12
	Gonococcal virulence.	
1.3.1.	Introduction	14
1.3.2.	Experimental gonococcal infection in animals	15
1.3.3.	Experimental gonococcal infection in man ...	16
	Host response to gonococcal infection.	
1.4.1.	Humoral response	22
1.4.2.	Cellular response	25
CHAPTER 2.	Materials and Methods	
2.1.1.	Organisms	27
2.1.2.	Cultivation	27
2.1.3.	Viable counting	28
2.1.4.	Preservation of Neisseria gonorrhoeae ...	28

Page No.

2.1.5. Standardisation of the growth phase and viability of gonococci 29

2.2.1. Human sera 30

2.2.2. Production of rabbit antisera 31

2.2.3. Absorption of sera 31

2.2.4. Column chromatography 32

2.3.1. The bactericidal test 32

2.3.2. Control for anticomplementary activity ... 34

2.4.1. Preparation of gonococcal lipopolysaccharide 34

2.4.2. Sensitisation of sheep red cells with gonococcal lipopolysaccharide 35

2.4.3. Preparation of glutaraldehyde fixed red cells 35

2.4.4. The haemagglutination test 36

CHAPTER 3. The cultivation and preservation of Neisseria gonorrhoeae.

3.1.1. Introduction 37

3.1.2. Evaluation of media for the enumeration of viable gonococci 37

3.1.3. The survival of gonococci in various diluents 39

3.1.4. The growth of gonococci in broth culture ... 40

3.2.1. The preservation of gonococci in liquid nitrogen. Introduction 43

3.2.2. Choice of a protective agent for freezing subcultured gonococci 44

3.2.3. Preservation of virulent gonococci 46

CHAPTER 4. The nature and heterogeneity of the antigens and antibodies involved in the serum bactericidal reaction against N.gonorrhoeae.

4.1.1. Introduction 49

Page No.

Factors influencing the survival of gonococci in normal human serum.

4.2.1. The effect of fresh and heated normal human serum on the survival of N.gonorrhoeae ... 49

4.2.2. A repeat experiment with additional strains of N.gonorrhoeae 50

4.2.3. The effect of the addition of homologous antibody on the survival of a gonococcus resistant to normal human serum 50

4.2.4. The effect of varying concentrations of complement and lysozyme 53

The heterogeneity of the antigens involved in serum killing

4.3.1. The sensitivity of 60 strains of N.gonorrhoeae to serum killing 55

4.3.2. The effect of serial subculture on the resistance of gonococci to serum killing 60

4.3.3. The sensitivity of 'typed' gonococcal strains in the bactericidal test 60

4.3.4. The involvement of more than one immunodeterminant in serum killing 61

4.3.5. The distribution amongst other bacterial species of antigens involved in the serum killing of N.gonorrhoeae strain G1 65

The heterogeneity, origin, and nature of the natural antibodies active in serum killing.

4.4.1. The distribution of gonococcidal natural antibodies in normal human sera 65

4.4.2. The origin of natural antibodies bactericidal for gonococci 65

4.4.3. The bactericidal activity of normal human serum IgG and IgM fractions 68

The nature of the antigens involved in serum killing.

4.5.1. Stability to heat and trypsin 69

Page No.

4.5.2. The participation of lipopolysaccharide in serum killing... 71

4.5.3. The amount of G1 lipopolysaccharide required to inhibit the serum killing of N.gonorrhoeae strains G1, G11 and G29 73

CHAPTER 5. Serological properties of gonococcal lipopolysaccharide.

5.1.1. Introduction 77

5.1.2. The choice of lipopolysaccharide for the haemagglutination test 77

Specificity.

5.2.1. Reactivity of gonococcal lipopolysaccharides with antisera to other bacterial species 80

5.2.2. Group specificity of gonococcal lipopolysaccharide 80

5.2.3. Serological specificity of Thomas and Mennie's extract 84

5.2.4. Comparison of the results of the haemagglutination and bactericidal tests 86

5.2.5. Intra Neisserial specificity of G1 lipopolysaccharide 89

5.2.6. Specific inhibition of the haemagglutination reaction with simple sugars 91

Detection of gonococcal antibodies.

5.3.1. The antibody response to gonococcal lipopolysaccharides 93

CHAPTER 6. A virulence attribute of gonococci lost on subculture.

6.1.1. Introduction 104

6.1.2. The recovery of gonococci from urethral exudates 105

Comparison between host grown gonococci and the same strain after subculture.

6.2.1. The effect of passage in vitro on the susceptibility of exudate and subcultured gonococci to serum killing 106

Page No.

6.2.2. The susceptibility of exudate gonococci to killing by human polymorphs 108

6.2.3. The sensitivity of exudate and subcultured gonococci to a pooled antigonococcal serum 110

6.2.4. The effect of exudate on the serum killing of a streptomycin resistant gonococcal strain, G11 112

6.2.5. The effect of growth phase on the serum killing of subcultured gonococci 115

Analysis of urethral exudate by immunodiffusion.

6.3.1. The presence of serum bactericidal components in urethral exudate 117

CHAPTER 7. The maintenance and assessment of gonococcal virulence in vitro.

7.1.1. Introduction 122

7.2.1. Susceptibility of gonococci of different colonial types to serum killing 122

7.3.1. The multiplication of gonococci in the chick embryo 127

Attempts to maintain gonococci in experimental animals.

7.4.1. Choice of laboratory animal 130

7.4.2. Growth of gonococci in the mouse peritoneum 130

7.4.3. The possible use of immunosuppressant drugs for modifying the resistance of mice to experimental gonococcal infection 131

CHAPTER 8. Discussion.

Antigenic structure 137

Virulence 142

Page No.

Appendix 1 151

Appendix 2 153

References 155

Publications 166

TABLES

		Page No.
Table 1	Quantitative evaluation of media for the viable counting of gonococci	38
Table 2	Survival of gonococci in DSB-s broth at various temperatures	41
Table 3	Survival of gonococci in Dulbecco's balanced salt solution at various temperatures	41
Table 4	The effect of different concentrations of glycerol and dimethylsulphoxide (DMSO) on the survival of 6 strains of N.gonorrhoeae frozen in liquid nitrogen. Recovery is expressed as a percentage of the original inoculum.	45
Table 5	The effect of different processing methods before freezing on the percentage recovery of gonococci frozen in urethral exudate	47
Table 6	The survival of gonococci in fresh and heated normal human serum	51
Table 7	Repeat of the previous experiment with three additional strains of N.gonorrhoeae ...	52
Table 8	The effect of varying concentrations of complement and lysozyme in the presence of a constant antibody concentration on the serum killing of N.gonorrhoeae G1	56
Table 9	Serum killing of Reyn's strains of N.gonorrhoeae	62
Table 10	Bactericidal cross reactions of absorbed gonococcal antisera with strains of N.gonorrhoeae ...	63
Table 11	Bactericidal reactions of absorbed gonococcal antisera with strains of N.gonorrhoeae - a repeat experiment	64
Table 12	Absorption of bactericidal antibodies from normal human or immune rabbit serum by Neisseria species, Escherichia coli and Pseudomonas aeruginosa	66
Table 13	The ability of normal human serum immunoglobulins G and M, with complement, to kill N.gonorrhoeae strain G2	70

Page No.

Table 14 Nature of the gonococcal antigens subserving the bactericidal reaction 72

Table 15 Antisera giving haemagglutinin titres of less than 40 with G1, G2 and G36 lipopolysaccharide coated red cells 81

Table 16 Antisera giving positive haemagglutinin titres to G1, G2, G22, G36 or G38 lipopolysaccharide coated red cells 83

Table 17 Haemagglutination titres of immune antigonococcal sera tested against sheep red cells sensitised with gonococcal lipopolysaccharides ... 85

Table 18 Serological specificity of Thomas and Mennie's extract compared with the phenol water lipopolysaccharide from the same gonococcal strains 87

Table 19 Comparison of the haemagglutination and bactericidal tests 88

Table 20 Intra-Neisserial specificity of absorbed anti G1 serum in the haemagglutination and bactericidal tests 90

Table 21 Effect of neutralisation with sugars on the haemagglutination titre of antibodies to gonococcal lipopolysaccharides 92

Table 22 Concentration of various sugars required to inhibit the agglutination of G36 lipopolysaccharide coated red cells by anti G37 94

Table 23 Statistical analysis of data in Fig. 10 ... 97

Table 24 Geometric mean haemagglutination titre of male patients' sera for G1, G2 and G36 lipopolysaccharide coated red cells as a function of the duration of infection 101

Table 25 Survival of exudate and freshly subcultured gonococci after incubation for 60 min. in a normal serum (MW) and the homologous patients' sera 109

Table 26 The survival of exudate gonococci after incubation for 60 min. with patients' serum and polymorphs 111

Table 27 The effect of complement and a pooled immune serum on the viability of exudate and subcultured gonococci 114

Page No.

Table 28 Comparison of the effect of growth phase on the killing of subcultured gonococci by a normal serum (MW) and homologous patients' sera ... 118

Table 29 Killing of gonococci of different colonial types by normal and immune sera 124

Table 30 Pathogenicity of gonococci via the chorioallantoic route for the 8 day chick embryo 129

Table 31 Survival of gonococci in the mouse peritoneum within 'Visking' sacs 132

Table 32 Survival of gonococci in the mouse peritoneum within Millipore chambers, 0.45μ 133

FIGURES

Page No.

1. Number of cases of gonorrhoea dealt with for the first time in England and Wales from 1940 4

2. Growth kinetics of 3 strains of Neisseria gonorrhoeae in biphasic flasks 42

3. The effect of varying concentrations of homologous antiserum on the complement-mediated killing of strain G1 54

4. Frequency distribution of percentage of organisms killed in 210 bactericidal tests with Neisseria gonorrhoeae using 1 normal human serum (MW) and 4 immune rabbit sera 57

5. Sensitivities of 60 strains of Neisseria gonorrhoeae to a normal human serum (MW) and 10 immune rabbit sera 59

6. Strain specificity of the bactericidal antibodies to Neisseria gonorrhoeae in normal human sera 67

7. Reduction of bactericidal and haemagglutinating activity following absorption with lipopolysaccharide coated red cells 74

8. Inhibitory effect of different concentrations of G1 lipopolysaccharide on the killing of N.gonorrhoeae strains G1, G11 and G29 by complement and anti G1 serum 76

9. Localised Shwartzman response in a rabbit. This demonstrates the endotoxic activity of a phenol-water extract of strain G36 79

10. Agglutination of gonococcal lipopolysaccharide coated red cells by normal and patients' sera 95

11. Comparison of the haemagglutination titres obtained for the same sera using different lipopolysaccharides ... 102

12. The effect of patients' serum on the corresponding strains of N.gonorrhoeae both in the native exudates and after subculture 107

13. The effect of polyvalent rabbit antiserum with human serum as a source of complement on strains of N.gonorrhoeae both in the native exudate and after subculture ... 113

Page No.

14. The effect of rabbit antiserum plus complement on a streptomycin resistant strain of N.gonorrhoeae (G11) in the presence of urethral exudate. Viable counts were made on media containing 25µg/ml streptomycin 116

15. Qualitative analyses of urethral exudate by immuno-diffusion and immunoelectrophoresis 119

16. Cortisol acetate induced wasting disease in neonatal mice 135

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ABBREVIATIONS

NHS	Normal human serum.
PBS	Phosphate buffered saline.
DSB-s	A broth prepared according to Difco's Dextrose Starch Broth formula, but omitting the starch.

CHAPTER 1 Review of the Literature.

The gonococcus as a successful pathogen.

1.1.1. Historical background.

A sexually transmitted disease resembling gonorrhoea was described in Leviticus (circa 1500 BC), whilst Hippocrates (400 BC) warned against the results of 'an excess of the pleasures of Venus'.

The bacterial aetiology of gonorrhoea was established in 1879 by Neisser who described a 'gonococcus' associated with the discharge from patients with gonorrhoea. The organism was first cultured by Lestikow and Loffler in 1882 and its pathogenicity demonstrated by human inoculation soon afterwards (see section 1.3.3.).

1.1.2. Incidence of Gonorrhoea.

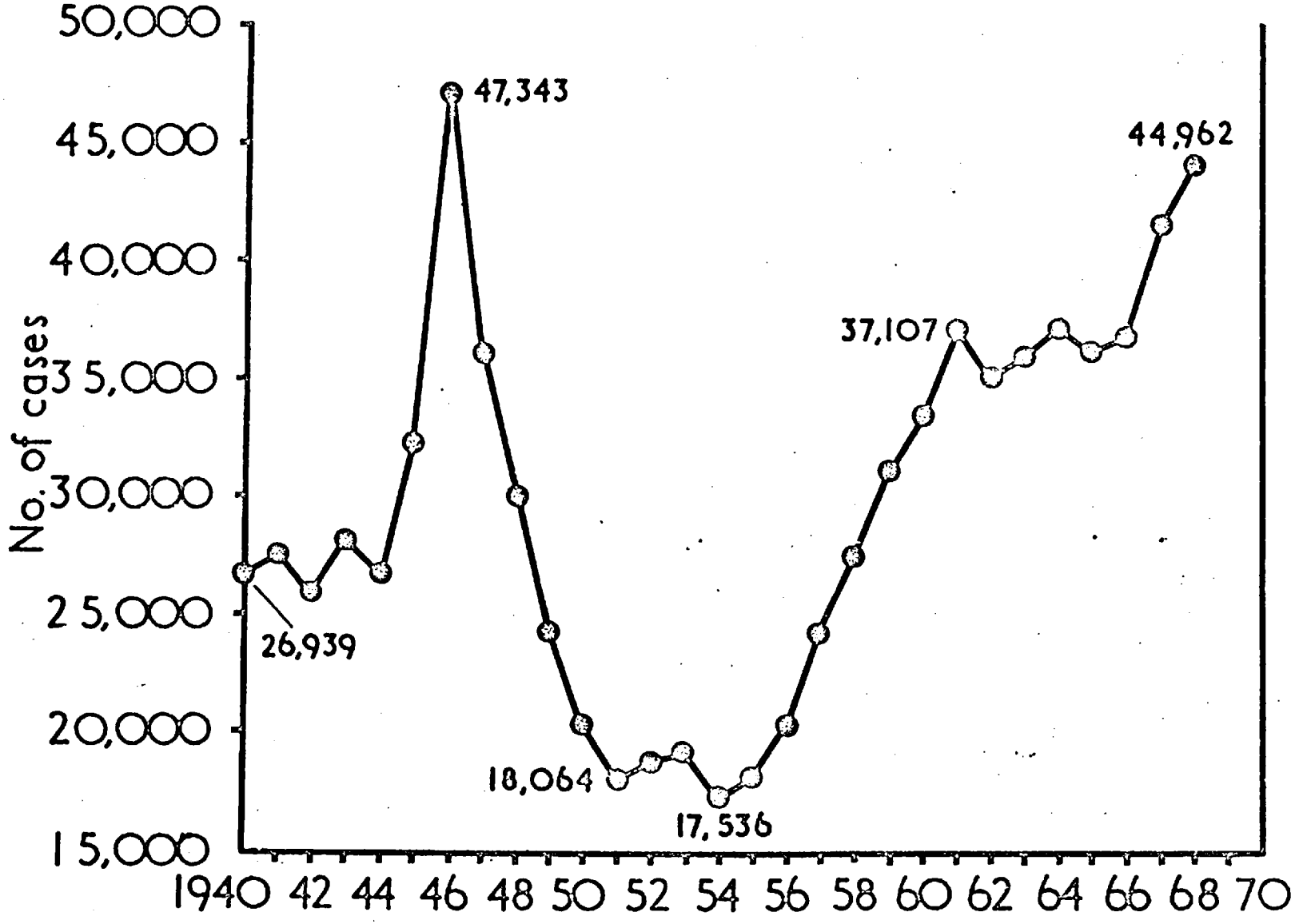
Although gonorrhoea is an ancient disease it was expected that the introduction of antibiotics would bring it under control. Fig. 1 shows that this expectation has not been fulfilled. In England and Wales the incidence of new cases of gonorrhoea is still increasing. The world wide incidence in 1963 was estimated at 60-65 million (WHO, 1963) but this estimate has recently been increased to 100 million (Catterall, 1969).

The success of the gonococcus has frequently been attributed to factors like increasing sexual promiscuity and population mobility (Willcox, 1969) however, the incidence of gonorrhoea is also correlated with the rate of increase of the population belonging to the most frequently infected age groups (Rose and Williams, Personal communication). Such factors fall outside medical control. Control of the

Fig. 1.

Number of cases of gonorrhoea dealt with for the first time in England and Wales from 1940.

(from King, A.J. (1970) Br. med. J., i, 451-457).


50,000
45,000
40,000
35,000
30,000
25,000
20,000
15,000
No. of cases
47,343
44,962
37,107
26,939
18,064
17,536
1940 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70

disease therefore depends on a better understanding of the pathogenicity and antigenic structure of the organism. Ultimately it is hoped this would lead to the development of improved diagnostic procedures and prophylactic immunisation.

1.1.3. The clinical manifestations of gonorrhoea.

In view of its ubiquity it is fortunate that gonorrhoea is a relatively benign disease. In the male the disease presents 2-3 days after infection as a urethral inflammatory discharge consisting of fluid and polymorphonuclear leukocytes with intra and extra-cellular gonococci. During this period the gonococci have penetrated the intercellular spaces of the urethral columnar epithelia, proliferating in the subepithelial connective tissue and provoking the inflammatory response (Harkness, 1948). If untreated the infection commonly persists but eventually cure is often spontaneous and the male asymptomatic carrier state is comparatively rare. Thus Pariser (1965) in a survey of 115 males who had been sexual contacts for at least six days of female patients with gonorrhoea, found that out of the 98 males who were asymptomatic 16% had gonococcal infections which were proven by culture. By contrast the majority of female patients are asymptomatic (WHO 1963; Catterall, 1969) although the organism may persist for long periods. This is of epidemiological importance since the asymptomatic carrier potentially provides a large reservoir of undetected infection in the population. In both sexes repeated infections frequently occur (Watt, 1958).

Gonorrhoea is normally regarded as a localised disease. However, this is not always the case. Local spread may occur involving the posterior urethra and epididymis in the male, or the Bartholins ducts and Fallopian tubes in the female. Salpingitis and epididymitis are particularly important complications because sterility may result, (Rees and Annels, 1969). In rare cases disseminated blood borne infections may occur giving rise to skin lesions or arthritis (Keiser et al., 1968).

The antigenic structure of Neisseria gonorrhoeae.

1.2.1. Introduction.

It is difficult to present a coherent picture of the antigenic structure of gonococci because no such picture exists. Since the 1940's there has been relatively little work on gonococcal antigens, and it may well be true that for no other bacterial pathogen of comparable importance is there so little knowledge of its antigenic structure. It is therefore proposed to point out some of the problems which have hindered research on this topic.

1.2.2. The heterogeneity and instability of gonococcal antigens.

No unanimity exists as to the degree of antigenic heterogeneity amongst gonococci. There seems to be general agreement that no consistent distinction can be drawn between them on the basis of agglutination or complement fixation tests (Stokinger et al., 1944c; Reyn, 1949b and c) with unabsorbed gonococcal antisera. However it

is possible to distinguish between them using absorbed gonococcal antisera. Thus Reyn (1949 a, b and c) was able to divide gonococci into a number of types on the basis of their reaction in the complement fixation test with variously absorbed gonococcal antisera. Several of the type antigens involved were coupled together whilst one of them occurred particularly frequently. However, difficulties were encountered due to many of the absorbed sera being anticomplementary, whilst strains which according to their origin ought to have been identical, e.g. simultaneous cultures of urethral and cervical strains from the same patients, often did not belong to the same serotype.

Wilson (1954) investigated the reactivity of absorbed gonococcal antisera in the agglutination test and postulated the presence of 4 group and 4 type specific antigens. Some of these were shared with the meningococcus (Wilson, 1956). Unfortunately, the usefulness of the technique was limited by the fact that many gonococci are autoagglutinable or inconsistent in their ability to absorb out agglutinins.

A further problem is that gonococci may alter their antigenic structure with subculture. Caspar (1941) stated that it became progressively more difficult to extract a specific polysaccharide as the number of subcultures of a gonococcal strain increased. However, this observation was limited to only 1 strain. Similarly Chanarin (1954) found that the ability of freshly isolated gonococci to yield a polysaccharide erythrocyte-sensitising extract ceased completely when the organisms had been in subculture for three months. This

antigenic alteration, which was noted with a number of strains, was associated with a change in the colony forms from smooth to rough. In support of this Wilson (1954) found that gonococci were capable of gaining or losing type antigens with subculture.

The most convincing demonstration of an alteration in the antigenic structure would be if an associated change in the sugar or amino acid composition of the cell wall or surface antigens could also be shown. This has yet to be done. However, there is no doubt that gonococci rapidly alter their colonial morphology on subculture (Kellogg et al., 1963). It therefore seems probable that alterations in their antigenic structure may also occur. Studies on the antigenic structure of gonococci have largely been performed using strains maintained by subculturing. Since the gonococcus may be antigenically unstable it is clearly essential to use adequate preservation techniques so as to retain its original structure as far as possible.

1.2.3. The chemical nature of the type specific antigens of gonococci.

It seems evident that gonococci are antigenically heterogeneous. If these antigens could be chemically characterised some of the problems of analysing gonococci by conventional serological procedures might be avoided.

Caspar (1937) extracted gonococci with 10% sodium taurocholate followed by sodium hydroxide and obtained a type specific polysaccharide which gave specific, delayed skin reactions in patients with gonorrhoea. It seems probable that this material represents highly degraded

polysaccharide derived from the endotoxin. A 'nucleoprotein' prepared from the same extract was less specific and gave a positive skin reaction in normal subjects. These results were subsequently confirmed by Uroma (1943) who with a similarly prepared polysaccharide was able to divide 30 strains into 4 types by complement fixation. Boor and Miller (1944) prepared a 'gluco-lipoid' by the Boivin extraction with trichloroacetic acid. The complete complex which probably consisted of the endotoxic protein and lipopolysaccharide complex was relatively non specific. However, after hydrolysis the carbohydrate fraction was found, using a precipitin test, to possess greater specificity.

The gonococcus, like other Gram negative bacteria, possesses an endotoxin. In the Salmonellae a clear relationship has been demonstrated between the structure of the polysaccharide component of the endotoxin and its O antigen specificity. In addition, antibodies to the O somatic antigen as measured in the Widal test are of diagnostic value. Nevertheless, despite this precedence there has been little work on the structure and specificity of gonococcal endotoxin. It is probable that some of the antigens already described were variously denatured endotoxin components, but it is difficult to know what these authors were really working with.

Chanarin (1954) following the technique of Thomas and Mennie (1950) prepared a soluble polysaccharide extract of freshly isolated gonococci using 0.05 N sodium hydroxide. This extract directly sensitised red cells to the haemolytic activity of complement and gonococcal antibody. This permitted the division of gonococci into 2 types, one of which was

much more frequent than the other. Considerable cross reaction was shown with meningococci and at least 2 antigens were present. One of these was involved in complement fixation but did not, like the other, sensitise red cells. Chanarin considered these 2 antigens might be present on the gonococcal surface as a complex which became partially split during extraction.

Maeland (1966) investigated the diagnostic usefulness of Chanarin's extract, but found it reacted with 55% of control blood donor sera. In a subsequent study (Maeland, 1968) the specificity of this extract was compared with gonococcal antigens extracted with phenol water, trichloroacetic acid, or aqueous ether. Each of these preparations in rabbits, with the exception of the Chanarin extract, gave a positive adrenalin skin test indicating the presence of endotoxic activity. Antibody to gonococcal lipopolysaccharide could be removed by absorption with Chanarin's extract, however in addition the latter contained another immunodeterminant which could be destroyed with proteolytic enzymes. Both of these polysaccharide and protein determinants were present in the aqueous ether and trichloroacetic acid endotoxins. In the aqueous ether endotoxin these determinants are present as a tightly bound complex since they were not separable by gel filtration, ultra-centrifugation or electrophoresis (Maeland, 1969a) whilst in a precipitin test using whole gonococci as antigen an antiserum to the endotoxin only gave a single line (Maeland, 1969c). This therefore probably represents the entire protein-lipopolysaccharide complex. The presence of endotoxin antigenic sites and the lack of a positive

adrenalin skin test suggests that the Chanarin extract also consists of a protein-polysaccharide complex in which any remaining lipid has been detoxified by saponification with alkali. It is well known that alkali treated lipopolysaccharides lose their ability to produce a local Shwartzman response (Lüderitz et al., 1968).

At present none of the qualitative analyses of gonococcal endotoxin have revealed any components which are unique to the gonococci. Maeland (1969d) reported that the aqueous ether endotoxin consisted of 90% protein. By comparison with other bacterial endotoxins this seems extraordinarily high and may indicate the presence of protein contaminants. Analysis of the polysaccharide components was therefore difficult because it constituted only 3% of the preparation. Nevertheless it was an important component because of its high serological activity (Maeland, 1969a) and the presence of some type specific immunodeterminants which are apparently absent in meningococci (Maeland, 1969d). The protein component contained at least 16 amino acids (Maeland 1969b). However, it was less specific than the polysaccharide and shared immunodeterminants with the meningococci (Maeland, 1969d), supporting the work of Boor and Miller (1944) previously described.

Absorption experiments with intact gonococci designed to determine the nature of the type specific antigens in situ have given equivocal results. Reyn (1949) considered that both thermolabile and thermostable antigens could absorb out type specific antibodies in the complement fixation test. However, Danielsson (1965) reported that strain or type specific precipitating antibodies could largely be removed by absorption

with gonococci heated to 100°C, suggesting that only thermostable antigens were involved. It seems probable that as in the Enterobacteriaceae it is the lipopolysaccharide which carries the type specific immunodeterminants.

1.2.4. The evidence for capsular antigens.

The presence of capsular antigens in meningococci has encouraged a search for similar antigens amongst the gonococci. Deacon et al.(1959) re-examined the observation of Wilson (1954) that freshly isolated gonococci are frequently inagglutinable in antigonoccal sera. These workers proposed that gonococci possess a species-specific antigen analogous to the K antigens of Escherichia coli or the Vi antigens of Salmonella typhi. It was postulated that this antigen was rapidly lost on subculture and was associated with gonococcal virulence. Their evidence for this is as follows.

Antisera were prepared to formalised, heated (100°C) and autoclaved inagglutinable gonococcal cultures. It is not clear whether the antigens and antisera employed were homologous, nor were the lengths of heat treatment specified. Living and formalinised gonococcal cultures were poorly agglutinated by antisera to heat treated gonococci. However, they reacted with an antiserum to formalinised gonococci. Following absorption with gonococci heated at 120°C the antiserum to the formalinised organisms agglutinated living, formalinised and heated (100°C) gonococci. It did not agglutinate gonococci which had been heated to 120°C. These results are compatible with the existence of a K type antigen which prevents the agglutination of gonococci by antibody to somatic antigens

and which is stable at 100°C but destroyed by heating to 120°C (Deacon et al., 1959). Circumstantial evidence for the loss of the proposed K antigen on subculture derives from the observation that fluorochrome conjugated antisera to formalinised gonococci solidly stain gonococci in urethral pus or early subculture. However, this intense solid staining becomes patchy and dim as the age of the subculture increases (Deacon, 1961).

There is considerable reason for disputing the interpretation which Deacon and his colleagues gave to their results. If the postulated K antigen is destroyed by heating gonococci at 120°C it is difficult to see why an antiserum to such a preparation should be able to agglutinate gonococci heated to 100°C which therefore still possessed K antigen. Danielsson (1965) attempted to repeat Deacon's results, but was unable to confirm them. He found that antisera to autoclaved gonococci which should not have contained Deacon's K antigen were able to agglutinate formalinised, heated or autoclaved gonococci with equal facility. Both thermolabile and thermostable antigens took part in the fluorescent antibody test. Lind (1967) criticised Deacon's work on the grounds that they have not distinguished between inagglutinability due to K antigen, and the inagglutinability of gonococci in heterologous gonococcal antisera described by Wilson (1954) which could easily have been due to different type specificities. Although she was able to show that four strains of gonococci, two of them old laboratory strains, were inagglutinable in a homologous antiserum raised against formalinised or heated cultures, there was no evidence of species specificity. Furthermore, the majority

of freshly isolated gonococci were autoagglutinable. Finally Kellogg et al. (1963) from the same laboratory as Deacon, found that a strain of N. gonorrhoeae grown to produce maximal amounts of K was avirulent for human volunteers. The postulated K antigen therefore appears not to be associated with virulence.

It might be expected that the possible occurrence of capsular antigens could be investigated by electron microscopy. Unfortunately, the electron micrographs so far published were generally performed on old laboratory strains whose antigens and ultrastructure may well have altered on subculture (Reyn, 1963; Ovčinnikov, 1963, 1966; Fitz-James, 1964). Although the cell walls have a trilamellar appearance characteristic of the Gram negative bacterial cell wall, there was no evidence for the presence of a capsule. Using light microscopy several workers have reported seeing encapsulated gonococci in negatively stained preparations (e.g. Bernstein, 1941). Unfortunately the possibility that these are staining or fixation artifacts cannot be excluded. The existence of capsular antigens in gonococci must therefore be considered unproven.

Gonococcal Virulence.

1.3.1. Introduction.

The usual experimental approach to the study of microbial virulence is to select a susceptible animal model for study. Ideally the relative role of the various host and parasite factors involved in eliminating an avirulant organism, whilst permitting a virulent organism to multiply, are then elucidated. However, even under relatively ideal conditions

this approach does not always give unequivocal answers to the complex question of host-parasite relations. For example in the study of Salmonella typhimurium or S. enteritidis infection in mice, which offers the advantages of infection of the natural host using an organism which is readily cultivable and whose antigenic structure is comparatively well known, the relative role of humoral and cellular defences is still highly debatable (Blanden et al., 1966; Jenkin and Rowley, 1963). The study of gonococcal virulence has none of these advantages. As already reviewed gonococcal antigenic structure is relatively unknown. There is as yet no relevant experimental model for gonorrhoea in animals. No criterion of virulence exists which could be causually related to the organisms' pathogenicity. Finally, the most serious problem of all is that there is reasonable evidence to suggest that gonococci rapidly become avirulent on subculture. In this review of gonococcal virulence it is proposed to discuss the evidence for these assertions and finally to consider the host's response to gonococcal infection.

1.3.2. Experimental gonococcal infection in animals.

So far as is known there is no natural gonococcal infection of animals (Ayyangar, 1970). Since ethical and practical considerations severely restrict the use of human volunteers, there has been an enormous amount of work on the production of an experimental model of gonorrhoea in animals which has been comprehensively reviewed by Hill (1944). Unfortunately, a large part of this work may be discarded

because of inadequate bacteriological controls. Thus the demonstration of Gram negative diplococci in a smear from an animal previously inoculated with gonococci does not mean they are still viable. For the present purposes the demonstration of gonococcal infection requires at least evidence for the persistence, if not multiplication, of gonococci within the host a minimum of 24 hr. after inoculation. In the majority of experiments the gonococci used have been aged laboratory strains. Frequently those results which seemed most encouraging have not been repeatable in the hands of other workers. Amongst the more enlightened attempts cited by Hill (1944) for producing a gonococcal infection in animals comparable to that in man are the introduction of gonorrhoeal pus into: a) the urethra of rabbits, mice, guinea pigs, monkeys and rams by Finkelstein and Timochina, b) the vagina of rabbits, bitches and cats by Jost, and c) of cultured gonococci into the knee joints of dogs and rabbits by Partsh and Nagell. Hill et al. (1945) obtained bacteriologically proven prolonged survival of cultured gonococci on the vaginal mucosa of the prepuberal mouse using various agents such as beef plasma or mucin in an attempt to modify the host resistance.

The partial modification of the host resistance obtained by Hill and her colleagues with relatively inefficient agents points the way to the use of immuno-suppressive drugs for this purpose. So far as is known this has not yet been reported. The chief danger in this approach is that the resulting artificially induced infection may be completely irrelevant to the disease in man. This criticism almost certainly applies to the three techniques at present available for

establishing gonococcal infections in animals. The ready multiplication of gonococci inoculated into the anterior chamber of the rabbit's eye (Miller et al., 1945) or into the yolk sac, amniotic sac or chorio allantoic membrane of the fertile hen's egg (Hill and Pitts, 1939) are completely unlike gonococcal infection in man. They probably represent the growth of gonococci in a nutritionally rich medium endowed with relatively poor defence capabilities. Thus Walsh et al. (1963) claimed that the chorio allantoic cavity of the 8 day old embyonated hen's egg suppressed the growth of avirulent gonococci whilst permitting the multiplication of virulent gonococci. Gonococcal virulence was assessed by human inoculation. Unfortunately no re-examination of this potentially important observation has appeared in the literature. It is known that the chorio allantoic fluid of the 8 day chick embryo forms an excellent growth medium for gonococci (Chacko and Nair, 1966). The work of Karthigasu et al. (1965) with Escherichia coli and of Ueda et al. (1969) with meningococci suggest that at this age the embryo has poor defence capabilities. However, an experimental conjunctivitis produced by dropping large doses of gonococci onto the surface of the eye and sealing the lids shut with plaster apparently resembled gonococcal conjunctivitis in man (Miller et al.,1945).

In 1940 Miller and Hawk succeeded in infecting mice with gonococci by inoculating them intraperitoneally suspended in hog gastric mucin. The role of the mucin was not clear and the dose required in the majority of experiments was close to that producing shock due to endotoxin. However, 5 out of 70 strains were found in which the minimum lethal dose,

after an enormous number of mouse passages with subculturing in between, was less than 10^3 organisms (Miller, 1944). This infection clearly bears little relationship to gonorrhoea. However, if it could be made less tedious it might provide a model for the study of disseminated gonococcal infection.

1.3.3. Experimental infection in man.

The failure to develop an animal model for gonorrhoea has lead to the investigation of experimental gonococcal infection in human volunteers. Much of the earlier work has been reviewed by Hill (1943). As with the animal infections much of the work is inadequate from the bacteriological standpoint because gonococci were not isolated by culture from a presumed infection. Not surprisingly, nearly all of the 96 papers reviewed are based on very few patients. However, two papers cited by Hill, one by Bumm (1885) and the other by Menge (1893) are of interest in that they established the aetiology of gonorrhoea and are the only reports of adequately controlled infections of females with gonococcal cultures. Although it is difficult to draw any conclusions from such variable data, Hill considered that a higher rate of infection overall was achieved using gonorrhoeal exudate than with subcultured gonococci.

The most comprehensive study of the conditions necessary to establish infections in man is that of Mahoney et al. (1946) on 241 male prison volunteers. Using enormous doses of cultured gonococci which were 'obviously far more drastic than is possible during the usual female to male transmission of the disease' it was only possible to produce clinical

gonorrhoea in about a third of the volunteers. Nevertheless, the resulting urethral exudates from those infected were capable in 12 out of 15 cases of establishing infections on transfer to a fresh host. The actual dose of exudate gonococci was probably lower than the doses of cultured gonococci used. With cultured gonococci there was no correlation between the incidence of infection and such possible important variables as: the strain, the medium, the depth of urethral inoculation, the duration for which the inoculum was retained, the suspending medium or, within limits, the dose. In particular, there was no correlation with the freshness of isolation of the strain, since even primary isolates of gonococci were relatively avirulent. Overall this constitutes reasonable evidence that gonococci rapidly become avirulent on laboratory media.

It has been known for some time that gonococci in subculture have different colonial morphologies, and this has been associated with their virulence (Atkin, 1925; Caspar, 1941). Kellogg et al. (1963, 1968) who re-investigated this phenomenon, showed that gonococci can be divided into four different colonial types. When freshly isolated from urethral exudates they consisted almost entirely of colonial type 1 or 2 (Sparling and Yobs, 1967). These colonial types, unless selectively subcultured, dissociated on laboratory media into types 3 and 4. The ability to maintain the respective colonial types with subculture suggested that a genotypic change was probably involved. Inoculation of colonial types 1 or 4 of the same strain after 69 transfers on laboratory media resulted in the infection of 4 out of 4 volunteers using colonial type 1 organisms. However, none of a further 4 volunteers were infected with colonial type 4 of the same

strain. On the basis of these results it was claimed 'we are now in a position to study Neisseria gonorrhoeae organisms in their virulent form'. This interpretation has apparently received wide acceptance (e.g. by Cohen, 1967; Chacko and Nair 1969; WHO, 1965; Peacock and Schmale, 1969). However, there is evidence to suggest that colonial type 1 gonococci are not completely virulent. Firstly Kellogg's own data shows that after 38 transfers on laboratory media colonial types 1, 2, 3 and 4 of the same strain produced infections in all the volunteers tested (Kellogg et al., 1963). This shows that the number of transfers a given colonial type undergoes is important, since it was only after an indefinite number of transfers between 38 and 69 that a change occurred making colonial type 4 avirulent. Secondly, the fact that 1.5×10^{10} colonial type 1 organisms were required to produce gonorrhoea in volunteers (WHO, 1965) suggests that they are not very virulent. This dose has apparently not been significantly reduced since in 1969 it was still being described as 'large' (Kellogg and Thayer, 1969). Thirdly, Mahoney et al. (1946) in their experiments on human volunteers were unable to detect any correlation between the use of 'conical' or 'flat' colonial types and the incidence of infection. It seems probable from the descriptions that these represent Kellogg's colonial types 1 and 2, and 3 and 4 respectively. It is, however, possible that in Mahoney's experiments they had not undergone sufficient transfers on artificial media for the 'flat' colonial type to completely lose the little virulence it possessed. It therefore seems necessary to seek an alternative explanation for Kellogg's results.

One possibility is that gonococci of colonial types 1 and 2 possess a greater ability in vivo to revert to the fully virulent form than do types 3 and 4. Large doses of gonococci are therefore required to give sufficient bacteria time to revert despite the destruction of avirulent organisms by the host defences.

The inability to maintain virulent gonococci on laboratory media is clearly a major problem in studying the pathogenesis of gonococcal infection. At present the only method of recognising virulent gonococci is by the experimental infection of volunteers. However, in the host the infecting organisms are able to invade the columnar epithelia so that in the laboratory their virulence might be associated with their invasiveness for suitable tissue culture cell lines. As an example of this, the virulence of shigellae for the guinea pig and cynomolgus monkey has been associated with their ability to penetrate intestinal epithelial cells both in vivo (La Brec et al., 1964) and in tissue cultures (Ogawa et al., 1967). It is well known that cultured gonococci will infect and destroy many of the common tissue culture cell lines (e.g. Gavrilescu et al., 1966; Gavrilescu and Lazăr, 1968; Davis and Pelczar, 1967). Unfortunately, none of these studies used cultures of those cell types which are invaded in the natural infection (Harkness, 1948) despite the availability, e.g. of adult conjunctival cell lines. Nevertheless, Kenny and Sparkes (1968) found that a material released into the medium in which Hela cells were growing, permitted the growth of two strains of colonial type 1 gonococci whilst suppressing the growth of the colonial type 3 forms. Chemically this material was similar to the 'retine'

described by Szent Györgyi et al. (1967) which retards the growth of normal cells, and appears to be a glyoxal. Methyl glyoxals up to C_{13} inhibited bacterial growth in low concentration but like the material Kenny and Sparkes described were inhibited by cysteine. However, Szent Györgyi reported that retine was absent from cancer cells, whilst HeLa cells were originally derived from a carcinoma.

Host response to gonococcal infection.

1.4.1. Humoral response.

In order to survive in the host a pathogenic microorganism must be able to resist its defence mechanisms, as well as being able to invade the hosts' tissues and make use of the nutrients available. It now seems likely that antibodies are produced at some stage in response to gonococcal infection. However, they have chiefly been examined from the point of view of their possible diagnostic usefulness.

The present method of detecting gonococcal antibody is the gonococcal complement fixation test (Price, 1930). However, despite the development of an improved antigen only 18.2% of positive results were obtained with the sera of patients with culturally proven gonorrhoea (Labzoffsky and Kelen, 1961). Consequently alternative diagnostic procedures have been advocated. Reising and Kellogg (1965) described a phenol extract of a colonial type 1 gonococcus which reacted with 62% of sera overall from male and female patients with proven gonorrhoea. A control group of 22 sera from laboratory personnel did not react. This antigen was probably lipopolysaccharide. However, it has now been discarded in favour of a

new preparation, 'A' antigen, of unknown chemical nature obtained by DEAE-Sephadex fractionation of gonococcal 'protoplasm' (Schmale et al., 1969). The 'protoplasm' consisted of the supernatant obtained after centrifuging at 36,000 g a suspension of gonococci which had been fractionated in a Ribi cell press (Martin et al., 1969). In a complement fixation test the 'A' antigen reacted with 71.6% of sera from infected females, 20.4% of sera from infected males, and no sera from individuals presumed free from gonococcal infection (Reising et al., 1969). However, although less specific than the purified 'A' antigen, the crude 'protoplasm' was weight by weight serologically more active. An apparently different antigen of unknown chemical nature has been described by workers from the same laboratory (Lee and Schmale, 1970). This antigen, 'B' antigen, was obtained by sedimentation at 4°C from a solution of lyophilised gonococcal 'protoplasm' in tris buffer and saline. In a slide flocculation test it reacted with sera from 86% of female or 68% of male patients. Reactions were also noted with 12% of control sera from presumed uninfected subjects.

The most sensitive test for the detection of gonococcal antibody so far described is that of Chacko and Nair (1969). These workers prepared a polysaccharide antigen, which they presumed was lipopolysaccharide but which gave a negative Shwartzman reaction. In a microprecipitin test gonococcal antibody was detected in sera from 60% of patients with a urethral discharge of 1-3 days, or in 95-100% of patients with a discharge of longer than 3 days. There was little or no reaction with normal sera.

The work of Cohen et al. (1969) suggests that diagnostically useful

antibodies may also be directed against themolabile, presumably protein antigens. They found that in volunteers experimentally infected with colonial type 1 gonococci the earliest significant antibody response was directed solely against heat labile (121^{o}C) antigens and was largely associated with IgG. Nevertheless, the large dose of gonococci required must have provided greater antigenic stimulation than the natural infection.

Prior to this work, which was published during the concluding stages of the present study, it was not clear whether with such a localised, relatively benign infection as gonorrhoea, there would be an antibody response soon after the infection. From the standpoint of serodiagnosis these results are encouraging. However, there is clearly a pressing need for an independent laboratory to compare the sensitivity and specificity of the various tests advocated using the same panel of patients or normal sera. Furthermore, the localised nature of gonococcal infections suggests there is a need to investigate the possibility of a localised immune response to gonorrhoea, which might perhaps involve secretory IgA.

Gonococcal antibodies may be diagnostically useful, but there is little evidence to suggest they serve a protective function. Mahoney et al. (1946) found that 56 out of 131 volunteers who had no previous history of gonorrhoea could be experimentally infected with gonococci. However, only 26 out of 108 volunteers who had had previous gonococcal infections could be subsequently experimentally infected. On a chi squared test the probability of these results being due to chance is less than 1%. Although these results could mean that there is a short

lived protective antibody response, they can also be explained by the partial replacement of the susceptible columnar epithelium by squamous cells which occurs after gonococcal infection (Harkness, 1948). Certainly the high frequency of multiple infections (Watt, 1958) suggests that they have little protective value. Similarly, the enormous world-wide incidence and high infectivity of gonorrhoea (Holmes, 1970) suggests that the natural antibodies active in serum killing (Cohen, 1967) also have little protective role.

1.4.2. Cellular response.

The function of the polymorphonuclear leukocytes in the inflammatory response is also uncertain. It has been suggested that gonococci survive within human polymorphs (Scherp, 1955; Smith, 1968) although no evidence was cited. Spink and Keefer (1937) added gonococci to the washed cells from whole blood suspended in saline and recovered live bacteria after 15 min. incubation. Since phagocytosis was apparent in stained smears these workers concluded that the polymorphs were unable to significantly kill gonococci. However, it could be that 15 min. is insufficient time to detect a significant change in the total number of bacteria with the procedures used. Watt (1970) from this laboratory found the half-life of cell associated gonococci to be 25 min. when incubated with human polymorphs compared to 5-10 min. with guinea pig polymorphs. Lysosomal cationic proteins (Zeya and Spitznagel, 1968) provided a possible mechanism for killing gonococci. Nevertheless, this difference did not explain the species-specificity and pathogenicity of N. gonorrhoeae, since in three

experiments the non-pathogenic N. pharyngis flavus was killed at the same rate as the gonococci. The role of humoral antibodies and polymorphs in the host response to gonococcal infection therefore remains unclear.

CHAPTER 2 Materials and Methods.

2.1.1. Organisms.

Strains of Neisseria gonorrhoeae designated G1, G3-G20, G22-G60 were isolated from male and female patients with gonorrhoea. They were identified as Gram negative, oxidase positive diplococci which fermented glucose but not maltose or lactose. N. gonorrhoeae strains G2, G21 and G91-G98 were obtained from the National Collection of Type Cultures, Colindale, and corresponded to NCTC 8375, 8448, 7129, 8254, 8676, 10022, 10223, 10224, 10226 and 10227, respectively. N. meningitidis NCTC 8339, N. pharyngis-siccus NCTC 4591, N. pharyngis-flavus NCTC 4590, N. flavescens NCTC 8263 and N. catarrhalis NCTC 3622 were also obtained from the same source. N. gonorrhoeae strains 1a, 2c, 42981, 12954, 11413 and 44341, designated G61R-G66R respectively, were kindly provided by Dr. Alice Reyn of the State Serum Institute, Copenhagen, and have previously been described by her (Reyn, 1949b and c). Pseudomonas aeruginosa R 133 and Escherichia coli WF 96 were from the culture collection at the Wright-Fleming Institute.

2.1.2. Cultivation.

Chocolate Serum Agar (supplied ready poured by Oxoid Ltd.) was initially used for the cultivation of gonococci. Subsequently this was replaced by a medium consisting of Difco GC base with 10% horse serum, 1% Difco yeast extract and 0.7% dextrose (Amies and Garabedian, 1967).

Cultures were incubated at 37^{o}C in a manometrically measured moist

atmosphere of 5% carbon dioxide in air contained in milk churns modified as described by Schaedler et al. (1965).

2.1.3. Viable Counting.

Serial ten fold dilutions of gonococci for viable counting were made in Difco's Dextrose Starch Broth prepared without the starch. This broth, subsequently referred to as DSB-s, permitted good survival of gonococci and was sufficiently anticomplementary to arrest the bactericidal activity of serum. 0.1 ml. volumes were spread over the surface of plates of Amies and Garabedian medium and incubated for 48 hr. Generally between 50-250 colonies per plate were counted. The moisture content of the plates was important. The most reproducible counts were obtained using plates which had been dried overnight at 37°C with the lid on, or alternatively for ½ hr. with the lid off. For gonococci in urethral exudates the highest counts were obtained using plates which had been freshly prepared and dried on the day of use.

2.1.4. Preservation of Neisseria gonorrhoeae.

To minimise the possibility of variations in antigenic structure or virulence occurring on subculture gonococci were preserved by freeze drying after the minimum number of cultures required to ensure purity (usually one or two). A fresh ampoule was used for each experiment.

A dense suspension of gonococci from an overnight culture on Amies and Garabedian was prepared in a medium comprising 3% w/v sodium glutamate and 5% w/v polyvinyl pyrollidone hydrochloride neutralised with sodium hydroxide (Obayashi et al., 1961). Approximately 0.2 ml volumes were

distributed in 100 x 6 mm freeze drying tubes and lyophilised in a Speedivac SP 5 centrifugal freeze dryer (Edwards High Vacuum Ltd.) for at least 3 hr. at a pressure of 30 microns or less of mercury.

This technique permitted the recovery of sufficient gonococci from an ampoule after overnight culture on Amies and Garabedian medium to provide a dense growth. However, a considerable number of gonococci died during lyophilisation, making it theoretically possible for the selection of atypical organisms to occur. An alternative technique giving 100% recovery of gonococci was therefore developed (see chapter 3).

Briefly, a dense suspension of an overnight culture of gonococci was prepared in 1% Difco proteose peptone no. 3 containing 8% v/v glycerol. 0.1 ml volumes of this suspension were dropped into liquid nitrogen. The resulting pellets were stored in screw capped polythene tubes (Sterilin Ltd.) in a liquid nitrogen container at -196^{o}C. When required pellets were rapidly thawed by dropping into 0.9 ml of proteose peptone broth at 37^{o}C. They were then cultured as previously described.

2.1.5. Standardisation of the growth phase and viability of gonococci.

An overnight culture of gonococci on Amies and Garabedian medium from a freeze dried ampoule was suspended in mineral salts solution (Dulbecco, Oxoid Ltd.) at 37^{o}C. The concentration of the suspension was adjusted so that a tenfold dilution in Dulbecco's solution had an optical density of 0.25 using the light grey filter of a Spekker absorptiometer. 0.2 ml of the original suspension was then inoculated into a modified version

of the biphasic flask described by Gerhardt and Hedén (1960). This consisted of a 100 ml screw cap bottle containing 10 ml of Difco Dextrose Starch Agar with an added 1% of agar. The Dextrose Starch Agar was then overlaid with 10 ml of DSB-s broth. The bottle was incubated on a shaking water bath for about 3 hr. at 37°C. At this stage varying slightly with the strain used the organisms were in the logarithmic growth phase at a viable concentration of approximately 10^9/ml (see section 3.1.4.).

2.2.1. Human Sera.

Normal human sera for use in the bactericidal test were obtained from nine volunteers comprising medical students or laboratory workers, with no history of venereal disease and who were not taking antibiotics. All of these sera were negative in the gonococcal complement fixation test. Blood was withdrawn into 50 or 100 ml syringes and distributed into sterile Universal bottles containing small beads. After gentle rotation for approximately 5 minutes the clot was removed and the serum separated by centrifugation at 2,000 g for 5 min. at 0°C. A portion of these sera were then pooled. They were then dispensed in 1 ml volumes and stored frozen at -60°C. Patients' sera were obtained from cases of proven gonorrhoea. Sera were thawed once only before use and then discarded. One of the normal sera (MW) was arbitrarily chosen for routine use in the bactericidal test. Complement titres for the pooled serum and serum MW were 50 and 52 $C'H_{50}$ units when measured by Mayer's method (Kabat and Mayer, 1961).

Control and patients' sera selected for use in the haemagglutination test are described in Chapter 5.

2.2.2. Production of rabbit antisera.

For the production of antigonococcal sera rabbits whose normal sera showed no detectable gonococcidal activity at a final concentration of 1:100 were selected.

Rabbits were injected intravenously with approximately 10^9 viable gonococci from an overnight culture on Amies and Garabedian medium on days 0 and 3. They were bled out on days 17-20. The sera were separated, sterilised by membrane filtration, tested for gonococcidal activity, and stored frozen at -20°C in 2 ml volumes.

2.2.3. Absorption of sera.

Approximately 2 ml of a 1:5 dilution of normal human serum (MW) or a 1:10 dilution of rabbit antigonococcal sera in Dulbecco's solution were mixed with 10% v/v of packed bacteria and incubated for 12 hr. at 4°C. The bacteria were removed by centrifugation at 2,500 g for 10 min., discarded, and the process repeated. After sterilisation by membrane filtration the sera were stored at 4°C. At the same time antisera were also absorbed with heterologous organisms to control the specificity of absorption.

To absorb antisera with gonococcal lipopolysaccharide 2 ml of a 1:20 dilution of rabbit anti G1 or G50 sera in Dulbecco's solution were mixed with 20% v/v of packed sheep red cells sensitised with either G1

or G36 lipopolysaccharides. After incubation for 4 hr. or overnight at $4^{o}C$ the cells were removed by centrifugation and the absorption repeated a further 4 to 7 times. Specificity of the absorption was checked by including a control tube in which the antisera were absorbed with unsensitised sheep red cells.

2.2.4. Column chromatography.

2 ml volumes of rabbit or human sera were dialysed overnight against 0.01M phosphate buffer, pH 7.5, and applied to a 30 x 1 cm column containing DEAE-cellulose. The column was then eluted at a flow rate of approximately 22 ml/hr. with the following buffers in order of application: 0.01 M phosphate pH 7.5, 0.1 M phosphate pH 6.5, and 0.3 M phosphate pH 6.5. 3 ml fractions were collected and their optical density at 280 nm measured on an SP 500 spectrophotometer. The serum fractions eluted by the above buffers were respectively IgG, impure IgA, and IgM. Each buffer was changed when the appropriate serum fraction as indicated by the O.D 280 peak had been obtained and the optical density had returned to the base line for at least 3 fractions. The fractions corresponding to a single peak were pooled and dialysed under reduced pressure against PBS pH 7.3 to a volume of 2 ml. After sterilisation by membrane filtration the serum fractions were stored at $4^{o}C$.

2.3.1. The Bactericidal test.

The bactericidal test was performed on gonococci standardised to the logarithmic phase of growth and a viable count of approximately 10^{9}/ml as

described in section 2.1.4. The organisms were then diluted 1,000 fold in Dulbecco's balanced salt solution at 37°C to a concentration of approximately 10^6/ml. This was sufficient to dilute out the anti-complementary effect of the broth. It was therefore possible to avoid washing the bacteria which had been found to reduce their viable count significantly. The Dulbecco solution also permitted adequate survival of gonococci at 37°C (see section 3.1.3.) and provided sufficient divalent cations (Ca^{++} 10^{-3} M., Mg^{++} 5×10^{-4} M.) to support the activity of complement.

0.25ml volumes of deep frozen normal human serum (MW) were used to provide complement and natural antibody for the bactericidal reaction. After dispensing on ice into sterile 3 x ½" tubes 0.1 ml of a 1:10 dilution of rabbit antisera in Dulbecco's solution was added where appropriate. The volume was then made up to 0.9 ml with Dulbecco's solution. Control tubes in which the complement had been inactivated by heating for 30 min. at 56°C were routinely set up in parallel. For five minutes prior to the addition of the gonococci the tubes were equilibrated to 37°C in a shaking water bath. 0.1 ml of Dulbecco's solution containing 10^5 organisms of the appropriate strain of gonococci were then added using automatic pipettes. Each tube therefore finally contained 13 $C'H_{50}$ units of complement as measured by Mayer's method. After mixing the tubes were shaken gently for 30 min. at 37°C in a shaking water bath. 0.1 ml samples were taken immediately after the addition of the gonococci from the control tubes containing inactivated serum. Thirty minutes later 0.1 ml samples were taken from all the tubes and immediately diluted 10 and 100 fold in DSB-s. 0.1 ml aliquots of

these dilutions were then immediately spread over the surface of plates of Amies and Garabedian medium before the next sample was taken. Generally 30 tubes were set up during each experiment. The tubes were therefore equilibrated, inoculated, sampled and the plates spread at 1 min. intervals. Strains were defined as sensitive if the test tube showed a reduction of 90% (i.e. 1 log. unit) or more in viable count in 30 min. when compared with the control lacking complement (see section 4.3.1.). The percentage of gonococci killed after adding specific antibody was calculated on the basis of the number of gonococci surviving after 30 min. incubation in 25% v/v of fresh serum MW.

2.3.2. Control for anticomplementary activity.

In order to determine whether absorbed or unabsorbed sera had any anticomplementary activity, the sample to be tested was mixed with normal serum (MW) in the proportions to be used in the bactericidal reaction. The tube was then inoculated with N. gonorrhoeae G2 and a bactericidal test carried out. This strain was killed by the natural antibody and complement present in serum MW. Any anticomplementary effect sufficient to interfere with the complement mediated killing of gonococci resulted in reduced killing of G2, when compared with that in a tube from which the absorbed serum had been omitted.

2.4.1. Preparation of gonococcal lipopolysaccharide.

Gonococcal lipopolysaccharides were prepared from strains G1, G2, G22, G36 and G50 by phenol water extraction using Westphal's method as

modified by Maeland (1968). Full practical details are given in Appendix 1. The purity and biological activity of the preparations is discussed in section 5.1.2.

2.4.2. Sensitisation of sheep red cells with gonococcal lipopolysaccharide.

In order to coat red cells with gonococcal lipopolysaccharide it was necessary to first treat the antigen with alkali. The required volume of lipopolysaccharide solution (1 mg/ml) was incubated at 37°C for 18 hr. with an equal volume of 0.02 N sodium hydroxide (Davies et al., 1958). After neutralisation with 0.02 N hydrochloric acid the solution was adjusted with PBS, pH 7.3 to the required concentration. Generally 12.5 µg/ml of the solution was required to optimally sensitise an equal volume of 2.5% v/v sheep red cells (see section 5.1.2.).

Sensitisation was performed by incubating the washed red cells in PBS, pH 7.3 for 1 hr. at 37°C with the lipopolysaccharide solution. The sensitised cells were washed three times in PBS and made up to a 0.5% v/v suspension in PBS containing a 1:250 concentration of normal rabbit serum which had been previously absorbed with normal sheep red cells.

2.4.3. Preparation of glutaraldehyde fixed red cells.

It was found that different batches of red cells varied in the degree to which they could be sensitised with the same lipopolysaccharide preparation. This complicated the comparison of haemagglutination experiments done at different times. This problem was overcome by preparing and storing large batches of normal and sensitised red cells which had been fixed with glutaraldehyde by the method of Bing et al.

(1967). The full procedure is given in Appendix 2. Sheep red cells treated in this way retained the same degree of sensitisation after storage for at least 6 months. The serological specificity of the lipopolysaccharide preparations remained the same. However, glutaraldehyde fixed cells gave slightly less distinct agglutination patterns.

2.4.4. The Haemagglutination test.

0.1 ml volumes of the appropriate 0.5% sensitised red cell suspension described in section 2.4.2. were added to an equal volume of serial two fold dilutions of antisera in a haemagglutination tray. The antisera dilutions were prepared in PBS, pH 7.3 containing a 1:100 concentration of normal rabbit serum which had been previously absorbed with sheep red cells. After mixing the trays were incubated for 1 hr. at 37°C, then overnight at 4°C. Results were read the following morning. Negative controls routinely included sensitised red cells in diluent alone, and unsensitised red cells in dilutions of the antisera.

C H A P T E R 3. The cultivation and preservation of Neisseria gonorrhoeae.

3.1.1. Introduction.

The use of experimental systems based on the viability of the organisms tested required the development of techniques for the enumeration and standardisation of viable gonococci. Adequate preservation techniques are also essential. This chapter outlines some of the experiments leading to the finalised methods of culture and preservation described in chapter 2.

3.1.2. The evaluation of media for the enumeration of viable gonococci.

Duplicate viable counts of 15 separate primary isolates of gonococci in PBS pH 7.3 were performed using four different media. These were chosen for their relative ease of preparation and consisted of chocolate serum agar (Oxoid), the media described by Thayer and Martin (1966) and Amies and Garabedian (1967), and Difco GC Base with 1% Bacto Supplement B (Kellogg et al., 1963). In addition, the effect on the recovery of gonococci of adding an antibiotic supplement to the medium described by Thayer and Martin (1966) consisting of a final concentration of 3 units/ml vancomycin and 7.5 µg/ml sodium colistimethate was also investigated. This antibiotic supplement is useful because it suppresses the growth of nearly all bacteria except the pathogenic Neisseriae.

Table 1 shows that on the basis of a paired data t test the number of gonococci recovered was significantly greater using Amies and Garabedian medium than with Thayer Martin's or Kellogg's media. Amies and Garabedian

Table 1

Quantitative evaluation of media for the viable counting of gonococci.

Gonococcal strain Number:	No. of gonococococci recovered (log_{10} viable count) from the following media:				
	Amies and Garabedian	Chocolate Serum Agar	GC Base + 1% Bacto Supp. B	Thayer Martin	Thayer Martin + antibiotics
1	7.06	7.06	6.93	6.81	6.81
2	7.18	7.22	6.99	7.14	7.08
3	7.64	7:63	6.36	7.55	6.95
4	7.56	7.57	7.56	7.58	7.56
5	7.53	7.47	6.91	6.03	5.70
6	7.48	6.91	6.32	6.36	6.00
7	7.06	7.11	7.06	6.91	6.83
8	6.94	6.91	6.86	6.88	6.80
9	7.36	7.23	7.15	7.09	6.93
10	7.17	7.18	7.02	7.14	7.08
11	5.81	5.76	5.67	5.62	4:30
12	6.46	6.32	6.43	6.62	6.04
13	6.92	6.85	6.91	6.82	6.78
14	7.48	7.45	7.31	7.41	7.33
15	7.08	6.96	6.99	6.91	6.88
Mean	7.12	7.04	6.83	6.86	6.6

Paired t tests

1) Amies and Garabedian medium significantly better than Difco GC base + 1% Bacto Supplement B or Thayer and Martin. t = 2.700, 2.240, d.f. = 14, P <1% and P >1.25% <2.5% respectively.

2) Amies and Garabedian medium is probably significantly better than Chocolate Serum Agar. t = 1.958, d.f. = 14, P > 2.5% <5.0%.

3) Addition of the antibiotic supplement to Thayer Martin medium significantly reduced the number of gonococci recovered. t = 2.773, d.f. = 14, P <1%.

medium was also probably significantly better than chocolate serum agar. Addition of the selective antibiotic supplement to Thayer-Martin base significantly reduced the number of gonococci recovered. However, this was to some extent due to the marked sensitivity of strains 5 and 11 to the supplement. In this connection Potuznik and Hausner (1969) found that there was only a small difference between the sensitivities of staphylococci and gonococci to vancomycin and considered there was a danger of inhibiting the growth of some of the pathogenic Neisseriae. These workers and Berger (1966) therefore advocated replacing the vancomycin with 5 or 10 µg/ml of lincomycin, respectively. On the basis of the results in Table 1 Amies and Garabedian medium was chosen for the quantitation of viable gonococci.

3.1.3. The survival of gonococci in various diluents.

In practice it was found to be not always convenient to plate immediately dilutions of gonococci for viable counting. The effect of various suspending media at different temperatures on the viability of gonococci was therefore examined.

Three strains of freshly isolated N. gonorrhoeae, G1, G9, G105, in the logarithmic growth phase were diluted in Dulbecco's solution to approximately 10^4/ml. 0.1 ml volumes of each suspension were inoculated into 3 tubes containing 0.9 ml of Dulbecco's solution and into 3 tubes containing the same quantity of DSB-s broth. One of these three tubes was incubated at $4^{\circ}C$, another at room temperature ($19^{\circ}C$) and a third at $37^{\circ}C$. Gonococcal survival was estimated by directly plating 0.1 ml

samples from the tubes immediately after inoculation and after 60, 120 and 180 min. incubation. The results (Tables 2 and 3) show that the survival of gonococci in DSB-s was excellent after 3 hr. incubation at all three temperatures, whereas in Dulbecco's solution survival at room temperature was markedly poorer than at $4^{o}C$ or $37^{o}C$.

Viable counts were therefore performed in DSB-s at room temperature whilst suspensions of gonococci prepared in Dulbecco's solution for the bactericidal test were maintained at $37^{o}C$ for not longer than 30 min.

3.1.4. The growth of gonococci in broth culture.

The use of broth cultures for standardising the growth phase and viable count of gonococci was investigated so as to avoid any variations in the bactericidal test due to inoculum size or growth phase. A total count of 4×10^{8} gonococci were inoculated into a biphasic flask prepared as described by Gerhardt and Hedén (1960) with the omission of the carbon dioxide. The flask was then incubated, with shaking, at $37^{o}C$. Viable counts were performed on the contents of the flasks immediately after inoculation, and after various incubation times. Fig. 2 shows the growth kinetics of three strains of gonococci in the biphasic flasks. It can be seen that after 2-3 hr. incubation the gonococci were in the logarithmic phase of growth with viable counts in the order of 10^{9}/ml. Further incubation resulted in the formation of sheets of gonococci so that, for viable counting, 1 colony forming unit would no longer correspond to a diplococcus. Omission of the starch or scaling down the size of the biphasic flask did not significantly alter the growth kinetics. The

Table 2

Survival of gonococci in DSB-s broth at various temperatures.

Strain No:	Incubation Time (min)	Log_{10} viable count of gonococci after incubation at:		
		4°C	19°C	37°C
G1	0	2.77	2.76	2.75
	180	2.73	2.59	2.90
Difference:		-0.04	-0.17	+0.15
G9	0	2.76	2.77	2.73
	180	2.72	2.53	2.90
Difference:		-0.04	-0.24	+0.17
G105	0	2.53	2.53	2.45
	180	2.45	2.43	2.48
Difference:		-0.08	-0.10	+0.03

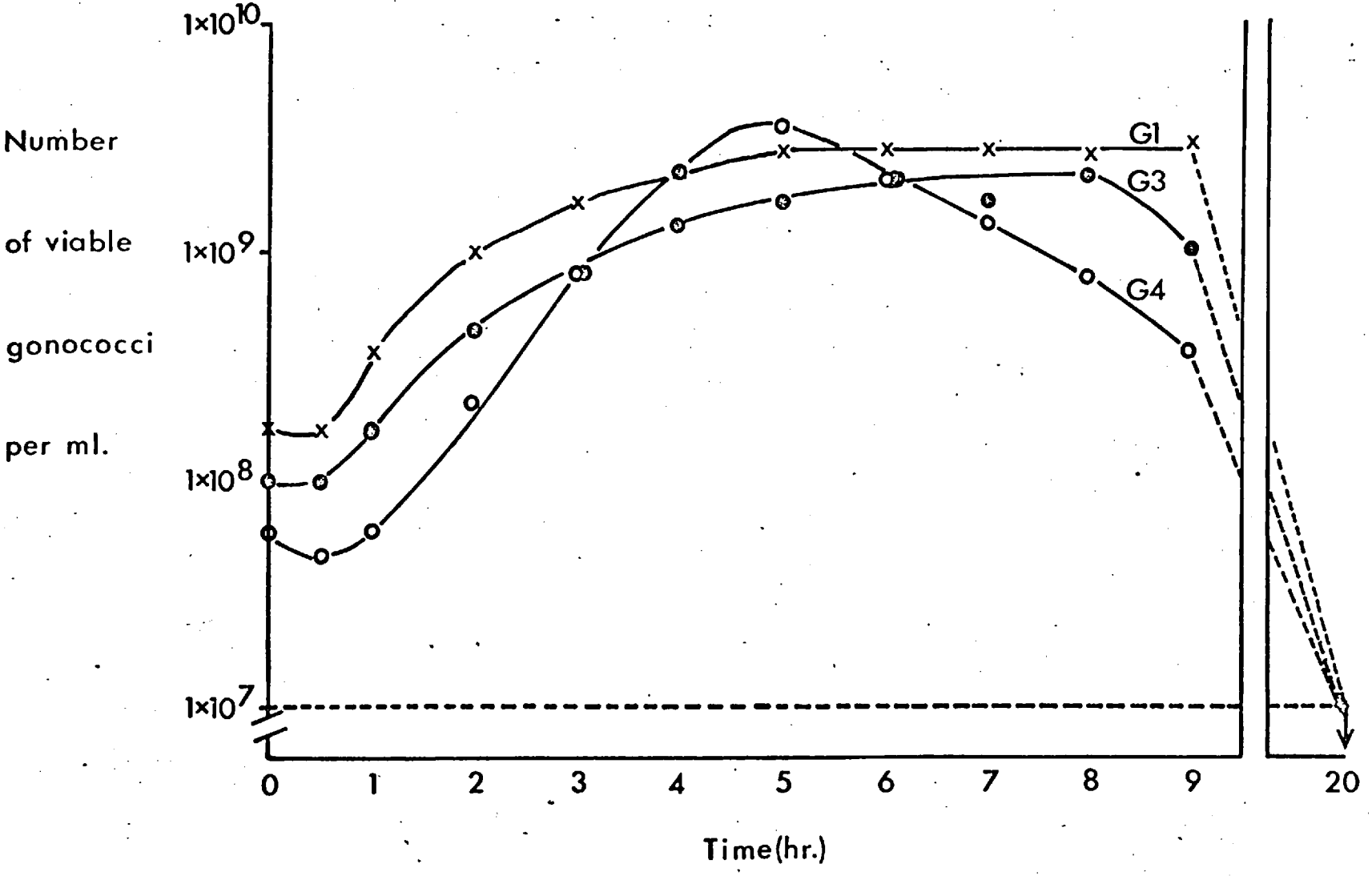
Table 3

Survival of gonococci in Dulbecco's balanced salt solution at various temperatures:

Strain No:	Incubation Time (min)	Log_{10} viable count of gonococci after incubation at:		
		4°C	19°C	37°C
G1	0	2.76	2.72	2.73
	180	2.38	1.00	2.28
Difference:		-0.38	-1.72	-0.45
G9	0	2.72	2.76	2.76
	180	2.47	1.00	2.15
Difference:		-0.25	-1.76	-0.61
G105	0	2.52	2.48	2.50
	180	2.40	1.60	1.78
Difference:		-0.12	-0.88	-0.72

Fig. 2.

Growth kinetics of 3 strains of Neisseria gonorrhoeae in biphasic flasks.


Number
of viable
gonococci
per ml.
1×10^10
1×10^9
1×10^8
1×10^7
G1
G3
G4
0
1
2
3
4
5
6
7
8
9
20
Time(hr.)

method outlined in section 2.1.5. was therefore finally adopted.

3.2.1. The preservation of gonococci in liquid nitrogen. Introduction.

It seems probable that gonococci alter both their virulence and antigenic structure with subculture (see chapter 1). A technique of preserving gonococci in the original exudate or after a minimal amount of culturing would therefore be invaluable. At present gonococci are preserved by lyophilisation (Chatelain, 1963; Fry, 1951; Greaves, 1960; Reyn, 1968). However, the recovery rate is usually under 5% of the number of viable bacteria in the original suspension (Brooks and Hedén, 1966). This low recovery rate makes it theoretically possible for the selection of atypical organisms to occur. It also makes it necessary to culture the contents of a freeze dried ampoule before they can be used.

Liquid nitrogen is now in routine use for the preservation of such delicate structures as tissue culture cells or erythrocytes. It might therefore be of use for the preservation of gonococci. Brookes and Hedén (1966) were able to recover between 21-34% of viable gonococci after freezing them in 15% glycerol broth in a 'Linde' controlled rate freezer. Reyn (1968) reported that she obtained an approximately 30% recovery of gonococci after freezing in broth alone. However, this was only a preliminary publication and no methods or supporting data were given. In neither of these papers was the possibility of preserving virulent gonococci in urethral exudate examined. The remainder of this chapter describes experiments leading to a simple method for freezing gonococci in liquid nitrogen giving 100% recoveries with subcultured

gonococci. An adaptation of the method for the preservation of the virulent gonococci present in urethral exudates is also described.

3.2.2. Choice of a protective agent for freezing subcultured gonococci.

Six strains of subcultured gonococci were chosen for these experiments. Four of these strains, Nos. 1, 2, 3 and 4, were fresh isolates consisting of colonial types 1 or 2. The remaining two strains were of colonial types 3 or 4. Each strain was grown overnight on Kellogg's medium and suspended to a concentration of approximately 10^8 viable organisms/ml. 100µl volumes of each suspension were distributed with an Eppendorf micropipette into 0.9 ml volumes of buffered Difco 1% proteose peptone No. 3 broth, with or without varying concentrations of dimethyl sulphoxide or glycerol (Kocka and Bretz, 1969) as protective agents (Table 4). After mixing, duplicate samples from each tube were snap frozen by dropping 0.1 ml volumes directly into liquid nitrogen. The resulting pellets were then immediately thawed by immersion in 0.9 ml volumes of DSB-s broth plus 5% w/v saponin at 37°C. Saponin broth was used in the first tube to make the experiment comparable with later experiments using gonorrhoeal exudate in which the saponin was found to give more reproducible viable counts. At the same time duplicate 0.1 ml samples were taken from the tube containing broth alone and inoculated into tubes of 5% saponin in DSB-s for determining the number of viable gonococci prior to freezing. Serial tenfold dilutions of the surviving gonococci were then prepared in DSB-s, duplicate 10µl aliquots from suitable dilutions spread over quarter sectors of Kellogg's medium, and the colonies appearing 48 hr.

Table 4 The effect of different concentrations of glycerol and dimethylsulphoxide (DMSO) on the survival of 6 strains of N. gonorrhoeae frozen in liquid nitrogen. Recovery is expressed as a percentage of the original inoculum.

Strain no.	SUSPENDING MEDIUM						
	Proteose peptone	8% glycerol peptone	16% glycerol peptone	24% glycerol peptone	6% DMSO peptone	16% DMSO peptone	24% DMSO peptone
1	13	98	58	76	37	56	15
2	50	98	100	115	98	87	23
3	18	132	112	98	48	21	16
4	27	63	76	72	57	29	27
5	25	140	100	135	56	50	20
6	22	115	107	105	66	49	13
Mean	26	107	92	100	60	49	19

Paired t tests

1) 24% glycerol is not significantly better than 8% glycerol as a protective agent., $t = 0.590$, d.f. = 5, P = >25%.

2) 8% glycerol is a significantly better protective agent than 6% DMSO., $t = 2.975$, d.f. = 5, P = > 1.25% < 2.5%.

later counted.

Table 4 shows that for the 6 strains overall the number of gonococci recovered after freezing in liquid nitrogen was considerably increased by adding protective agents to the broth. 8% glycerol was a significantly better protective agent than 6% dimethylsulphoxide. At higher concentrations dimethylsulphoxide is probably toxic, since the number of gonococci recovered after freezing in 24% dimethylsulphoxide broth was less than the number recovered after freezing in broth alone. Since there was no significant difference between the various concentrations of glycerol the lowest concentration was chosen. The recoveries of greater than 100% of the inoculum, obtained using glycerol as the protective agent, are probably due to the separation of diplococci or clumps of gonococci during freezing.

3.2.3. Preservation of virulent gonococci.

When gonococci in urethral exudates were homogenised with a Teflon grinder in 8% glycerol peptone there was no detectable loss on freezing (Table 5). However, if the exudates were suspended without homogenisation in 8% glycerol peptone and immediately frozen only 32% of the viable gonococci were recovered, suggesting that the glycerol was failing to penetrate the cellular debris and other materials present. As would therefore be expected, this recovery rate was considerably improved by increasing the incubation time of the gonococcal exudates in glycerol broth to 15 min. and raising the temperature to 37^{o}C. The addition of 5% saponin, which will rupture polymorphs without affecting the viability of gonococci (Watt, 1970) did not increase the number of organisms

TABLE 5

The effect of different processing methods before freezing on the percentage recovery of gonococci frozen in urethral exudate.

Homogenised in 8% glycerol peptone	Suspended in 8% glycerol peptone	Incubated 15 min at 37°C in 8% glycerol peptone	Incubated 15 min at 37°C in 8% glycerol peptone containing 5% saponin
102	33	59	40
105	36	40	36
150	36	66	66
121	40	86	97
185	15	138	62
Mean: 133	32	79	60

recovered.

The high recovery rate of gonococci frozen in liquid nitrogen allows the direct use of frozen organisms as the starting inoculum for the bulk growth of gonococci. It also permits the preservation of gonococci both in urethral pus and after primary subculture for use in studies on gonococcal virulence. It also seemed possible that this technique might form the basis of a simple method for transporting infected specimens from areas lacking adequate laboratory facilities.

Specimens of urethral pus were collected on charcoal impregnated swabs from 20 patients with acute gonorrhoea. The infected swabs were broken off into screw capped polythene ampoules (Sterilin Ltd) containing 0.4 ml of 8% glycerol peptone. After remaining at room temperature for periods of one to three hours, the swabs were cultured and then frozen by immersing the ampoules in liquid nitrogen. Between 7-10 days later the ampoules were thawed by immersion in a 37^{o}C water bath and the swabs were again cultured. Gonococci were recovered from all 20 of the specimens frozen.

Since biological activity is believed to be zero at the temperature of liquid nitrogen (-196^{o}C) and this temperature is also below that at which ice crystals enlarge, there is no reason to suspect any loss in viability after storage for an indefinite period of time (Brookes and Hedén, 1966). This, together with the high recovery rates obtained, makes it very unlikely that any selection of atypical gonococci can occur.

CHAPTER 4. The nature and heterogeneity of the antigens and antibodies involved in the serum bactericidal reaction against N. gonorrhoeae.

4.1.1. Introduction.

Earlier work has shown that gonococci are serologically heterogeneous (see section 1.2.2.). However, by and large the antigens involved remain ciphers which might be important in the serodiagnosis of gonococcal infection but whose function and composition remain unknown. Investigation of the structure and function of gonococcal antigens has been made particularly difficult firstly, by the lack of a suitable experimental animal model and secondly, by the loss of virulence and antigenic instability of gonococci cultured in vitro. In addition, as was mentioned in section 1.2.2., the use of conventional procedures like complement fixation or agglutination for the study of gonococcal antigens is frequently difficult.

Ideally, antigens used for investigating the immune response in gonorrhoea should ultimately be chemically defined. Rather than performing a blindfold chemical dissection of the organism, it was decided to use the serum bactericidal reaction for studying the gonococcal antigens. It seemed possible that this might overcome some of the difficulties mentioned, whilst being more likely to reveal antigens of biological significance which could later be studied chemically.

Factors influencing the survival of gonococci in normal human serum.

4.2.1. The effect of fresh and heated normal human serum on the survival of N. gonorrhoeae.

Approximately 10^5 organisms of N. gonorrhoeae strains G1, G12 and G13,

in 0.1 ml of Dulbecco's solution were each added to tubes containing either 0.9 ml, i.e. 90%, of fresh normal human serum (MW), or serum which had been inactivated by heating at 56°C for 30 min. Viable counts were performed at 0, 15, 30, 60 and 120 min. The results (Table 6) showed that the survival of all three gonococcal strains was equally good in the presence or absence of complement.

4.2.2. A repeat experiment using additional strains of N. gonorrhoeae.

The resistance of the three gonococcal strains in the previous experiment might have been because the bactericidal reaction against gonococci is strain specific. A further possibility is that all gonococci may be inherantly resistant to killing by serum. The experiment was therefore repeated and a further three strains examined. The results of the previous experiment were confirmed, whilst two of the additional three strains were rapidly killed in the presence of complement (Table 7).

4.2.3. The effect of the addition of homologous antibody on the survival of a gonococcus resistant to normal human serum.

The results of the previous experiment suggested that gonococci might be resistant to normal human serum due to the absence of suitable antibody. To test this idea approximately 10^5 logarithmic phase gonococci (strain G1) in 0.1 ml of Dulbecco's solution were added to 0.9 ml of fresh or heated human serum containing final concentrations of between 1:10 to 1:10,000 rabbit anti G1 serum prepared as described in section 2.2.2. They were then incubated at 37°C and samples were removed for viable counting after 0, 30, 60 and 90 min. To test whether any of the antisera were anti-complementary a 0.1 ml sample was removed from each tube after 15 min.

TABLE 6

The survival of gonococci in fresh and heated normal human serum.

Heated Serum	Fresh Serum	Strain	Log_{10} Viable Count after Incubation for: 0	15	30	60	120 min.
+	-	G13	5.50	5.50	5.47	5.69	5.99
-	+		5.49	5.40	5.36	5.43	5.64
+	-	G12	5.40	5.34	5.53	5.52	6.11
-	+		5.30	5.52	5.54	5.54	6.03
+	-	G1	4.90		4.90	4.95	
-	+		4.87		4.70	4.40	

TABLE 7

Repeat of the previous experiment with three additional strains of N. gonorrhoeae.

Heated Serum	Fresh Serum	Strain	Log_{10} Viable Count after Incubation for: 0	15	30	60	120 min.
+	-	G1	5.15	5.11	5.11	5.23	
-	+		5.08	4.90	5.00	5.04	
+	-	G2	5.40		5.30	5.30	
-	+		5.26		2.00	<2.00	
+	-	G3	5.04		5.30	5.18	
-	+		4.95		<3.00	<2.00	
+	-	G4	5.18		5.18	5.18	
-	+		5.08		4.85	4.90	
+	-	G12	5.37	5.34	5.48	5.57	
-	+		5.29	5.21	5.12	5.01	
+	-	G13	5.52	5.51	5.49	5.27	6.11
-	+		5.56	5.43	5.40	5.46	5.69

incubation, diluted in 0.9 ml of ice cold complement diluent (Oxoid) and maintained for not longer than 30 min. in flake ice before assay. Doubling dilutions of 0.5 ml volumes of the sample in complement diluent were prepared and incubated for 30 min. at 37°C with 0.1 ml of a 1% sheep red cell suspension sensitised with rabbit anti sheep red cell haemolysin (Staynes). The end point was taken as the last tube showing partial haemolysis.

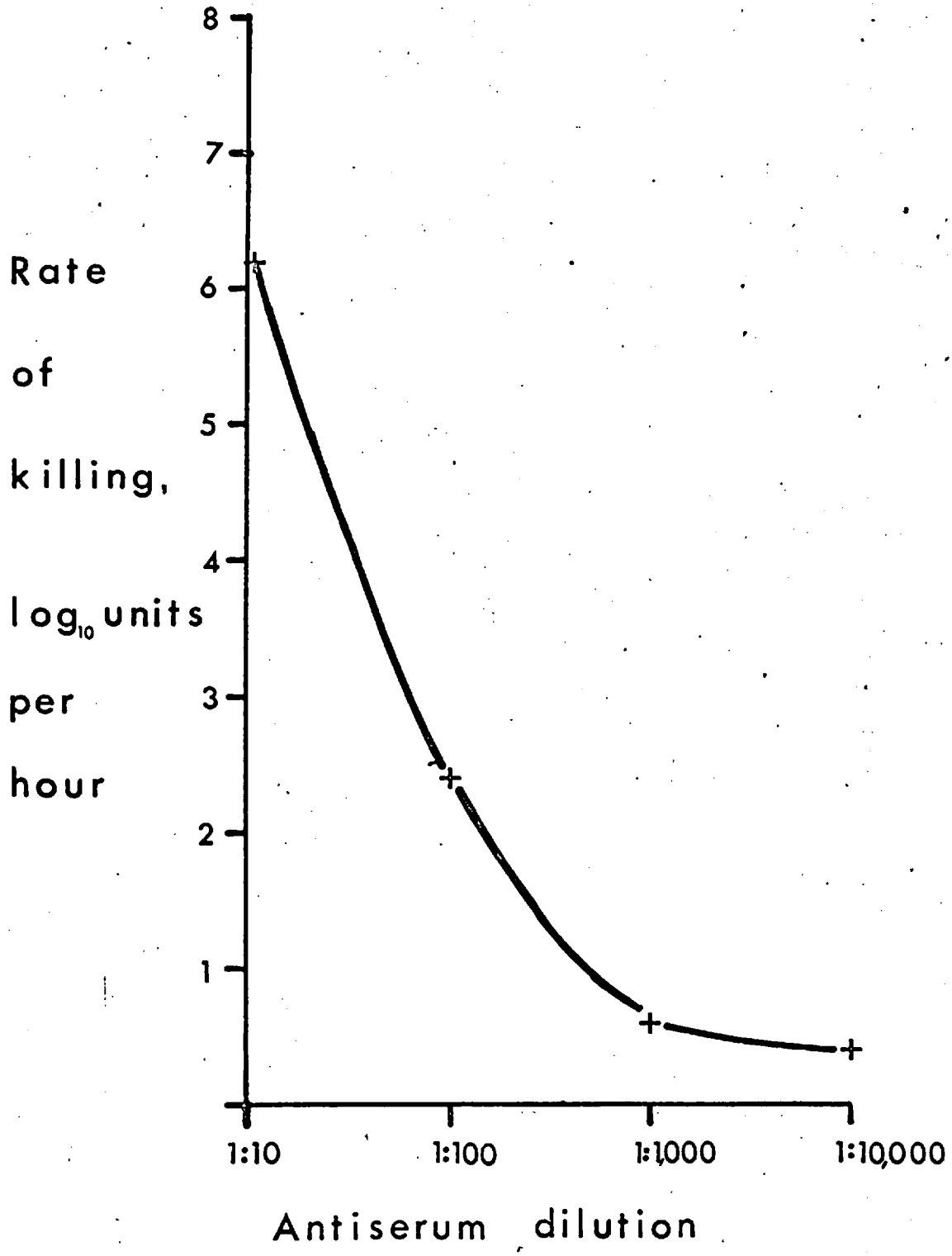
No killing occurred in the absence of complement with even the highest concentrations of antiserum. However, as is shown in Fig. 3, detectable killing occurred in the presence of complement with as little as a 1:10,000 final concentration of anti G1 serum. The rate of killing was markedly limited by the concentration of antiserum, no prozonal effect such as that observed by Cohen et al. (1967) being observed at higher antiserum concentrations. No anticomplementary effect was detected with any of the concentrations of antiserum. Unless otherwise stated antisera were therefore used at a concentration of 1:100 for investigating the antigens involved in the bactericidal reaction and the experimental tubes were incubated for 30 min.

4.2.4. The effect of varying concentrations of complement and lysozyme.

Tubes were prepared containing a final concentration of 1:100 rabbit anti G1 serum with varying volumes of fresh normal human serum and the final volume adjusted to 0.9 ml with Dulbecco's balanced salt solution where necessary. After equilibrating to 37°C 0.1 ml volumes of Dulbecco's solution containing 10^5 logarithmic phase gonococci (strain G1) were added

Fig. 3.

The effect of varying concentrations of homologous antiserum on the complement-mediated killing of strain G1.


Rate of killing, log10 units per hour
8
7
6
5
4
3
2
1
1:10
1:100
1:1,000
1:10,000
Antiserum dilution

to the tubes and incubated on a shaking water bath at 37°C. Viable counts were done on the number of gonococci present at the start of the experiment and after 30 min. incubation. Strain G1 is not killed by the complement and antibody present in serum MW. Therefore, the results (Table 8) show that in the presence of a constant amount of antibody the killing was unaffected by reducing the serum concentration to 10% v/v. This suggests that both complement and any lysozyme required were not rate limiting at this concentration. However, for strains which are sensitive to the bactericidal action of normal human serum, dilution of the serum, by diluting the appropriate antibody, might affect their rate of killing. For examining the antigens involved in serum killing a concentration of 25% v/v serum was used. However, in experiments designed to test the significance of serum killing in the hosts' defences to gonococcal infection, much higher concentrations of serum were employed (see chapter 6).

The heterogeneity of the antigens involved in serum killing.

4.3.1. The sensitivity of 60 strains of N. gonorrhoeae to serum killing.

Sixty strains of N. gonorrhoeae whose origins, isolation and preservation by lyophilisation have been described in chapter 2, were screened in the bactericidal test as outlined in section 2.3.1. using the control normal human serum (MW) as a source of complement and antibody. Strains which showed 90% (i.e. 1 log unit) or more loss when compared with the control lacking complement were defined as sensitive on the basis of the frequency distribution of the percentages of organisms killed in 210 tests

TABLE 8

The effect of varying concentrations of complement and lysozyme in the presence of a constant antibody concentration on the serum killing of N. gonorrhoeae G1.

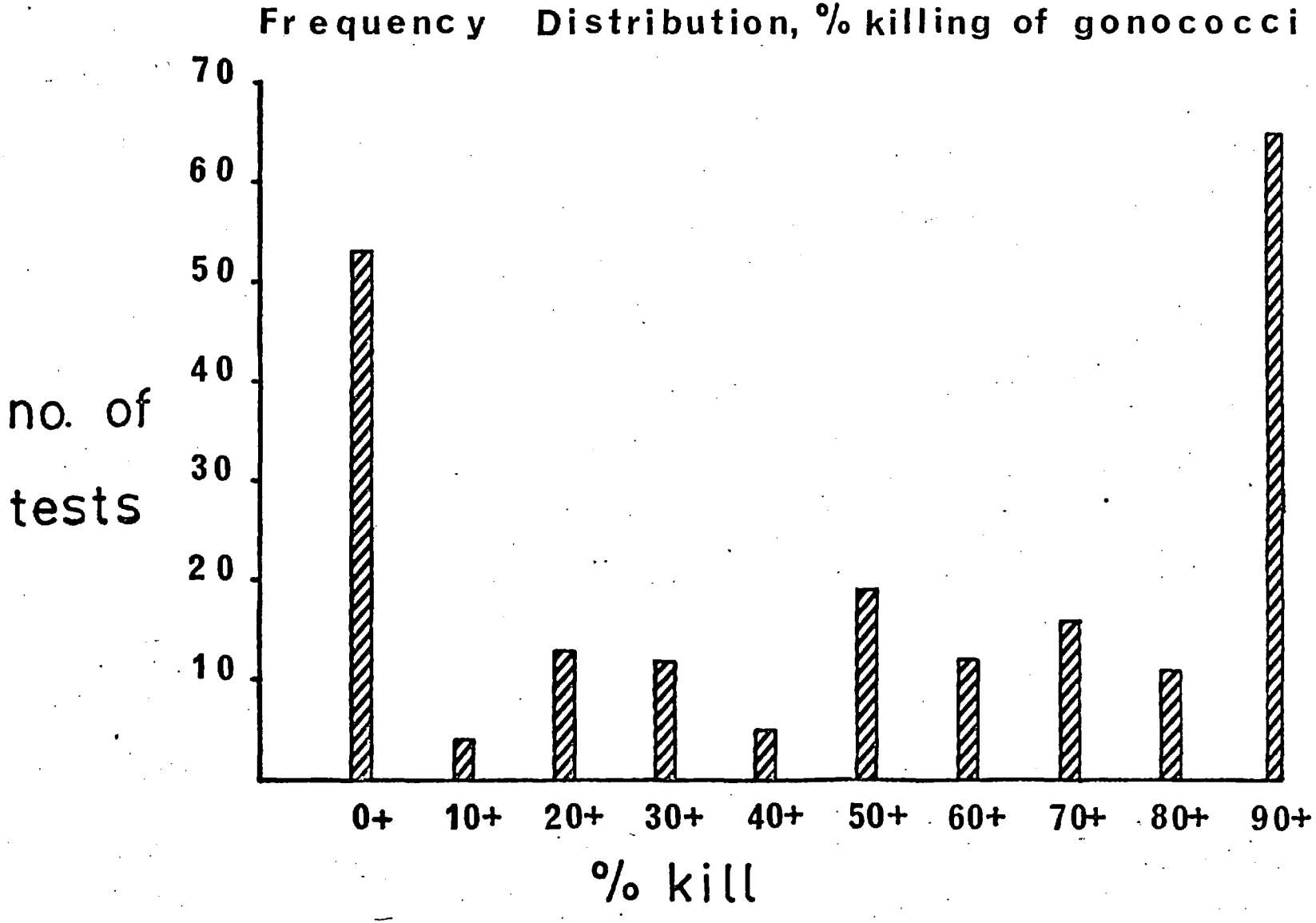
% v/v serum MW in Dulbecco's solution	Concentration of anti G1	Viable count after incubation for: 0	30 min.	% Kill
80% Control [a]	1:100	1×10^4	9×10^3	10[b]
80%	1:100	1×10^4	$<1 \times 10^1$	>99
50%	1:100	1×10^4	$<1 \times 10^1$	>99
25%	1:100	1×10^4	$<1 \times 10^1$	>99
10%	1:100	1×10^4	1×10^1	>99

a = serum MW inactivated at 56°C for 30 min.

b = within experimental error.

Fig. 4.

Frequency distribution of percentage of organisms killed in 210 bactericidal tests with Neisseria gonorrhoeae using 1 normal human serum (MW) and 4 immune rabbit sera.


Frequency Distribution, % killing of gonococci
no. of tests
70
60
50
40
30
20
10
0+
10+
20+
30+
40+
50+
60+
70+
80+
90+
% kill

on 60 strains (Fig. 4).

Strains which were resistant to the bactericidal activity of normal human serum were retested after the addition of immune serum to a final concentration of 1:100. The results are shown in Fig. 5. Clearly the antigens involved in serum killing are very heterogeneous. Nevertheless, it is possible to divide gonococci into four groups according to the antibodies required for serum killing to occur.

Group 1 comprises strains which were killed by the natural antibodies present in serum MW.

Group 2 strains were killed by the addition of anti G1, G2, G3 or G4 sera.

Group 3 comprises strains which were killed in the presence of anti G37 or G50 sera, but not anti G1, G2, G3 or G4.

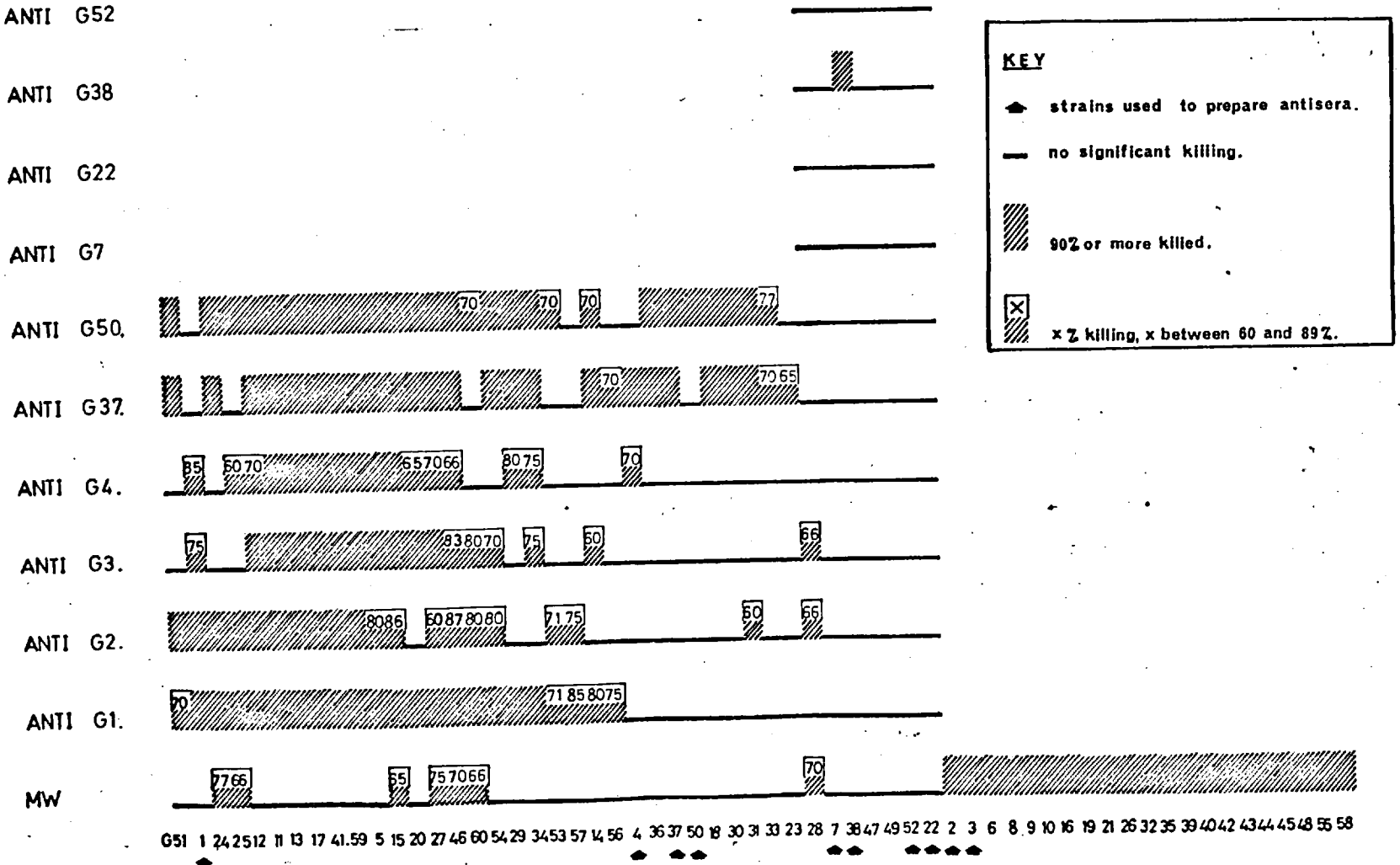
Group 4 strains were resistant to anti G1, G2, G3, G4, G37 or G50 sera and also to anti G7, G22, G38 and G52. Strains G22 and G52 were fully resistant and G7 partially resistant to their homologous antisera.

Group 4 is of particular interest in that it includes some strains which were not killed even in the presence of their homologous antisera. Nevertheless, these same antisera were found to have bactericidal activity to other gonococcal strains (see section 4.3.3.). Strain G38 is at present included in group 4 even though it was killed by its homologous antiserum. Like G38 there are several other strains which do not fit clearly into this grouping. Thus strain G1 does not fit into group 2 because it is

Fig. 5.

Sensitivities of 60 strains of Neisseria gonorrhoeae to a normal human serum (MW) and 10 immune rabbit sera. Strains were defined as sensitive if the test tube showed a reduction of 90% (i.e. 1 log unit) or more in viable count in 30 min. when compared with the control lacking complement. Resistant strains were retested after the addition to the system of rabbit immune serum. The percentage of organisms killed by the immune sera is calculated from the number of gonococci surviving after 30 min. in 25% fresh serum MW alone. The key also applies to Fig. 6.

SERUM KILLING OF GONOCOCCI.



STRAIN NUMBER.

resistant to anti G3 serum, but neither does it fit into group 3 because of its resistance to anti G37 and anti G50. It is evident that additional groups or subdivisions could be defined with further work on the same lines.

4.3.2. The effect of serial subculture on the resistance of gonococci to serum killing.

Since gonococci are considered to be antigenically unstable (see section 1.2.2.) the possibility that the resistance of gonococci to serum killing might alter with subculture was investigated.

Strains G1 and G36, which are resistant to killing by normal human serum, were subcultured every 2-3 days for 6 months on Amies and Garabedian medium and periodically tested. There was no evidence to suggest any significant alteration in their resistance. Similarly, the proportion of sensitive to resistant strains amongst 9 aged laboratory strains of gonococci from the National Collection of Type Cultures, Colindale, i.e. $1:2.\overset{3}{\not{3}}$, was not significantly different to the proportion found in 58 fresh gonococcal isolates, i.e. 1:3.

4.3.3. The sensitivity of 'typed gonococcal strains in the bactericidal test.

Using a complement fixation test and absorbed gonococcal antisera Reyn (1949 b and c) was able to assign factor formulae to a number of gonococcal strains (see section 1.2.2.). It seemed possible that the factor antigens involved in complement fixation might be related to the antigens active in the bactericidal reaction. Dr. Alice Reyn kindly

provided lyophilised specimens of some of her strains which were tested as described in section 4.3.1. for their sensitivity to killing by normal human serum (MW) and the ten rabbit antisera (Table 9). No correlation was found between the results of the bactericidal tests and the factor formulae. Strains 2a and 42981 clearly fell into group 1, whereas strains 1a and 44341 would belong to group 4 except for the fact that they were sensitive to anti G1 and anti G2. It seems probable, therefore, that the antigens involved are different.

4.3.4. The involvement of more than one immunodeterminant in serum killing.

In an attempt to confirm the results of section 4.3.1. (Fig. 5) anti G1 serum was absorbed with strains G1, G29, G34 and G36. These strains were resistant to serum MW but, with the exception of G36, were killed by adding anti G1 serum. As expected, absorption of anti G1 serum with strains G1, G29 or G34, largely abolished its bactericidal activity. However, absorption with strain G36 surprisingly made the anti G1 serum ineffective against G29, less effective against G34, but left it fully effective against G1. This suggests that the results were not due to non specific absorption (see Table 10). To further ensure that these results were valid, the experiment was repeated using a different batch of absorbed sera. Comparison of Tables 10 and 11 shows that the results obtained were virtually the same. The inability of anti G1 serum after absorption with strain G36 to kill strain G1 but not G29 suggests that more than one immunodeterminant on a given organism is involved in serum killing. The nature of these is the subject of further discussion in chapter 8.

TABLE 9

Antiserum killing of Reyn's strains of N. gonorrhoeae.

	Per cent killed [a]				
Strain no:	1a	2c	42981	11413	44341
Factor formula:	V	V, IX	X,(VI),IX	III, VI	IX, XII
Antiserum Normal (MW)	60	>99	>99	0	70
Anti G1	92	ND	ND	>99	>99
" G2	50	"	"	>99	>99
" G3	10	"	"	75	0
" G4	0	"	"	90	0
" G37	0	"	"	90	50
" G50	0	"	"	96	50
" G7	0	"	"	97	50
" G22	0	"	"	95	83
" G38	0	"	"	95	15
" G52	0	"	"	95	0

a = Per cent killed was calculated by comparison with that produced by normal human serum.

ND = not done

TABLE 10

Bactericidal cross reactions of absorbed gonococcal antisera with strains of N. gonorrhoeae.

HEATED SERUM MW	FRESH SERUM MW	1:10 ANTISERUM	ABSORBED WITH	PERCENTAGE KILLING[b] (30 MIN.) OF STRAINS: G1	G25	G29	G34	G36	G41	G51	G2[a]
+	-	-	-	0	0	0	0	0	0	-25	-25
-	+	-	-	0	0	0	0	0	0	0	99
-	+	ANTI G1	-	99	97	98	98	0	99	99	99
-	+	ANTI G2	-	98	96	50	33	-33	66	43?	99
-	+	ANTI G3	-	ND	ND	90	93	0	ND	25	99
-	+	ANTI G4	-	ND	ND	90	93	50	ND	53	99
-	+	ANTI G1	G1	0	ND	ND	ND	ND	ND	ND	99
-	+	ANTI G1	G29	40	33	-25	-25	ND	-11	ND	99
-	+	ANTI G1	G34	0	-25	0	33	ND	0	ND	99
-	+	ANTI G1	G36	92	ND	0	70	ND	ND	ND	99
-	+	ANTI G2	G36	96	ND	ND	ND	ND	ND	96	99
-	+	ANTI G2	G51	40	77	ND	ND	ND	0	-25	99

ND = not done. a = anticomplementary control. b = Minus percentage represents percent growth.

TABLE 11

Bactericidal reactions of absorbed gonococcal antisera with strains of N.gonorrhoeae - a repeat experiment.

HEATED SERUM	FRESH SERUM	ANTISERUM 1:10	ABSORBED WITH	PERCENTAGE KILLING (30 MIN.) OF STRAINS: G1	G29	G36	G2[a]
+	-	-		0	0	0	-25
-	+	-		0	55	33	>99
-	+	ANTI G1		>99	98	0	99
-	+	ANTI G2		ND	67	-50	ND
-	+	ANTI G3		ND	95	-50	ND
-	+	ANTI G4		ND	96	50	ND
-	+	ANTI G1	G1	0	0	ND	99
-	+	ANTI G1	G29	50	0	ND	99
-	+	ANTI G1	G36	96	33	ND	>99

ND = not done. a = Anticomplementary control.

4.3.5. The distribution amongst other bacterial species of antigens involved in the serum killing of N. gonorrhoeae strain G1.

Anti G1 serum was absorbed with various bacteria and tested as a source of antibody for the serum killing of G1. With the exception of N.pharyngis-siccus NCTC 4591 and N.gonorrhoeae strain G2, none of the bacteria were capable of removing bactericidal antibody to G1, suggesting that at least some of the immunodeterminants involved in serum killing may be specific to the gonococcus (see Table 12).

The heterogeneity, origin and nature of the natural antibodies active in serum killing.

4.4.1. The distribution of gonococcidal natural antibodies in normal human sera.

Eight of the normal human sera described in section 2.2.1. were tested for bactericidal activity at 25% concentration against 5 gonococcal strains G2, G16, G40, G45, G55, which were sensitive to killing by natural antibodies in serum MW. A further 5 strains which had proved resistant to serum MW were similarly tested against a pool of equal volumes of the eight normal human sera. Fig. 6 shows that normal sera varied greatly in their ability to kill the 5 sensitive strains. None of these sera when pooled were capable of killing the 5 resistant strains.

4.4.2. The origin of natural antibodies bactericidal for gonococci.

Donors of normal human sera were selected on the basis of freedom from previous gonococcal infection. Since the gonococcus is not part of

TABLE 12

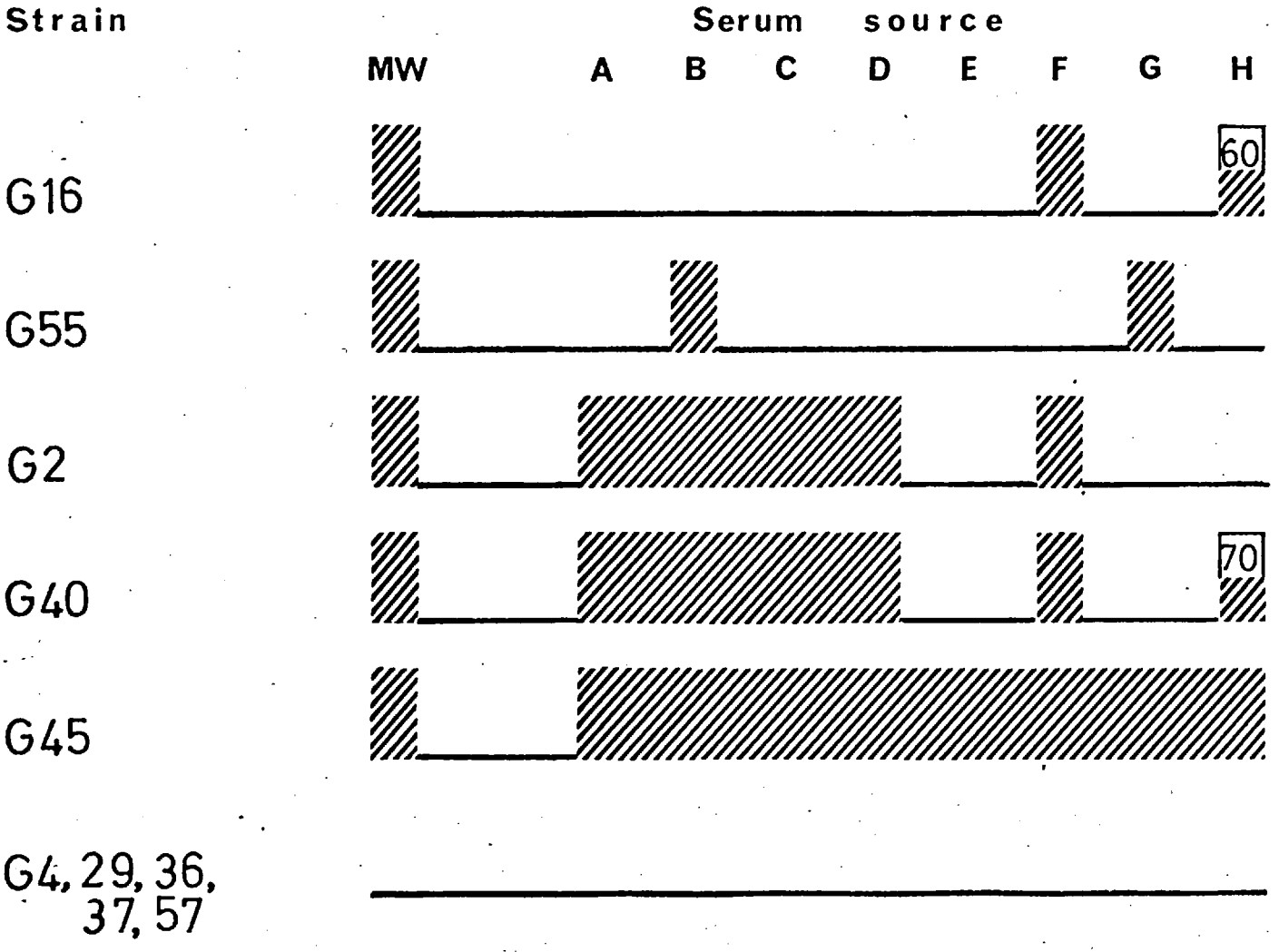
Absorption of bactericidal antibodies from normal human or immune rabbit serum by Neisseria species, Escherichia coli and Pseudomonas aeruginosa.

Serum absorbed with	Percent kill at 30 minutes of	
	Strain G2 tested with normal human serum (MW)	Strain G1 tested with rabbit anti G1 serum
Unabsorbed control	>90	>90
Neisseria gonorrhoeae G1	>90	0
" " G2	0	0
N.flavescens NCTC 8263	>90	>99
N.meningitidis NCTC 8339	66	>99
N.catarrhalis NCTC 3622	33	>99
N.pharyngis-flavus NCTC 4590	0	>99
N.pharyngis-siccus NCTC 4591	0	0
Escherichia coli WF 96	0	ND
Pseudomonas aeruginosa R 133	> 90	>99

Fig. 6.

Strain specificity of the bactericidal antibodies to *Neisseria gonorrhoeae* in normal human sera.

Normal bactericidal antibodies to Neisseria gonorrhoeae



the normal microbiota the question which arises is how have these natural antibodies originated? They might have been produced as a result of stimulation by related antigens in Neisseriae or other organisms forming part of man's natural flora. To examine this hypothesis a 1:5 dilution of normal human serum (MW) was absorbed with various bacteria which might conceivably be antigenically related. 0.2 ml volumes of the absorbed serum were then tested as a source of antibody for the serum killing of G2, a strain which is sensitive to killing by the natural antibodies in serum MW. 0.25 ml of a different normal human serum, serum G (Fig. 6) was used as complement source since it did not contain bactericidal antibody to G2. The results are shown in Table 12. Absorption with N.pharyngis-siccus, N.pharyngis flavus and N.flavescens which are commonly carried in the nasopharynx of man, or with E.coli WF96 (O7 K1 H6) which was isolated from urine, removed the bactericidal antibodies from serum MW. Surprisingly little effect was produced by a strain of N.meningitidis, although meningococci are antigenically related to gonococci (see chapter 1). Absorption of serum MW with a strain of Pseudomonas aeruginosa which is probably antigenically unrelated to the gonococcus, or with a strain of gonococcus, G1, which was resistant to killing by serum MW, did not affect its bactericidal activity for strain G2. Control absorption of serum MW with the sensitive strain G2 abolished the serum's bactericidal activity as expected.

4.4.3. The bactericidal activity of normal human serum I_gG and I_gM fractions.

2 ml of serum MW was fractionated by column chromatography on

DEAE-cellulose as described in section 2.2.4. The peaks corresponding to the IgG and IgM fractions were collected separately and reduced to the original volume of the serum by ultradialysis against PBS pH 7.3. 0.2 ml volumes of the whole serum, IgG and IgM fractions were each tested as sources of natural antibody in the bactericidal reaction against G2. Unfortunately, due to dwindling stocks of the appropriate normal human sera it was necessary to use as complement source a normal human serum, serum E (see Fig. 6) which had slight bactericidal activity for G2. The IgA fraction was not tested for bactericidal activity since by this fractionation technique it is invariably contamination with IgG and IgM. Although on a molecular basis IgM is regarded as being approximately 100 times more effective than IgG in the bactericidal reaction (Robbins et al., 1965; Daguillard and Edsall, 1968) whole serum and both the IgG and IgM fractions provided natural antibody for the serum killing of G2 (see Table 13). No contaminating immunoglobulins in the fractions were detected by Ouchterlony gel diffusion using anti human IgG or IgM sera (Hyland). However, the precipitin test is much less sensitive than the bactericidal reaction. In retrospect it would have been better to treat the IgG fraction with 2 mercapto-ethanol to destroy any residual IgM followed by dialysis to remove the 2 mercapto-ethanol. Unfortunately, the experiment could not be repeated because of the shortage of a normal human serum which would not kill strain G2.

The nature of the antigens involved in serum killing.

4.5.1. Stability to heat and trypsin.

Anti G1 serum was absorbed as described in section 2.2.3. with

TABLE 13

Ability of normal human serum immunoglobulins G and M, with complement, to kill N.gonorrhoeae strain G2.

Complement Source Normal human serum	Antibody Source Normal human serum serum MW	Immunoglobulin Fraction (of serum MW)	Log_{10} Viable gonococci after incubation at 37°C for		% Kill
			0	30 min.	
-	-	-	3×10^5	3×10^5	0
+	-	-	3×10^5	1×10^5	66
+	+	-	3×10^5	2×10^3	98[a]
+	+	IgM	3×10^5	4×10^3	97[a]
+	+	IgG	3×10^5	1×10^4	90[a]

a = percentage kill due to added antibody.

10% v/v of packed G1 which had been treated with heat or trypsin. 0.1 ml volumes of the absorbed antisera were then tested as sources of antibody in the bactericidal reaction using G1 as the test strain. Trypsin digestion was performed by heating 0.2 ml of packed gonococci at 50°C with 0.05% w/v of the 3 times recrystallised enzyme (Koch-Light, Ltd.) at pH 8.0 for approximately 2 hr. until no further solubleisation occurred (Hill, M.J., Personal communication). Trypsin and soluble materials were then removed by washing 4 times in PBS pH 7.3. The heat treatments consisted either of steaming packed gonococci for 1 hr., extracting them in boiling saline for 30 min., or autoclaving them at 15 p.s.i.g. (121°C) for 2 hr. As table 14 shows, bactericidal antibody to strains G1 and G29 was removed from anti G1 by absorption with trypsinised, heated (100°C) or autoclaved G1. These results were not due to non-specific absorption since control absorption of the antiserum with heat treated G36 (100°C) as expected removed bactericidal antibody to G29 but not to G1. The antigens involved in the serum killing of G1 and G29 were therefore highly resistant to heat and trypsin, suggesting that they were most likely not proteins but polysaccharides.

4.5.2. The participation of lipopolysaccharide in serum killing.

The resistance of the antigens involved in serum killing to heat and trypsin suggested that, as with other Gram negative bacteria, lipopolysaccharide might be involved. Antisera to strains G1 and G50 were absorbed 5 times with sheep red cells coated with purified lipopolysaccharides from strains G1 and G36 respectively. Absorption

TABLE 14

Nature of the gonococcal antigens subserving the bactericidal reaction.

Anti G1 serum absorbed with	Percentage killing in 30 min. of: G1	G29	G2[a]
Unabsorbed	>90	98	> 99
G1 live	0	34	> 99
" 100° 1 hr.	0	0	94
" 121°, autoclave, 2 hr.	0	78	99
" 100°, in saline, ½ hr.	0	56	99
" trypsin	0	0	96
G36 100° 1 hr.	>90	33	>99

a = anticomplementary control.

of anti G1 serum with red cells coated with the homologous lipopolysaccharides reduced its bactericidal activity for G1, and abolished it for G29 (Fig. 7). Absorption of anti G50 with red cells coated with G36 lipopolysaccharide partially removed bactericidal antibody to strains G1, G36, G37 and G50. Specific haemagglutinating activity of the absorbed anti G1 and G50 sera for red cells coated with G1 and G36 lipopolysaccharides respectively was almost entirely removed, even in those cases where bactericidal activity was still partially retained. The bactericidal and haemagglutinating activities of control sera absorbed with unsensitised sheep red cells were not significantly affected. The implications of these results and those of the following section, are discussed in chapter 8.

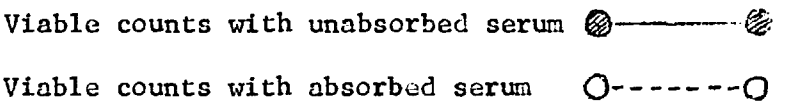
4.5.3. The amount of G1 lipopolysaccharide required to inhibit the serum killing of N.gonorrhoeae strains G1, G11 and G29.

In the previous section the lipopolysaccharide was, in effect, made insoluble by binding onto a carrier particle, in this case a red cell. This technique reduced the possibility of the lipopolysaccharide being anticomplementary, but it is difficult to quantitate the amount of lipopolysaccharide fixed to the cells. In order to determine the amount of lipopolysaccharide required to inhibit the bactericidal reaction the method had to be modified.

1 ml volumes of 1:10 anti G1 serum in Dulbecco's solution were neutralised with doubling dilutions containing between 40 and 1.25µg of purified G1 lipopolysaccharide. The neutralisation was performed by incubating the lipopolysaccharide-antiserum mixture at 37°C for 1 hr.

Fig. 7.

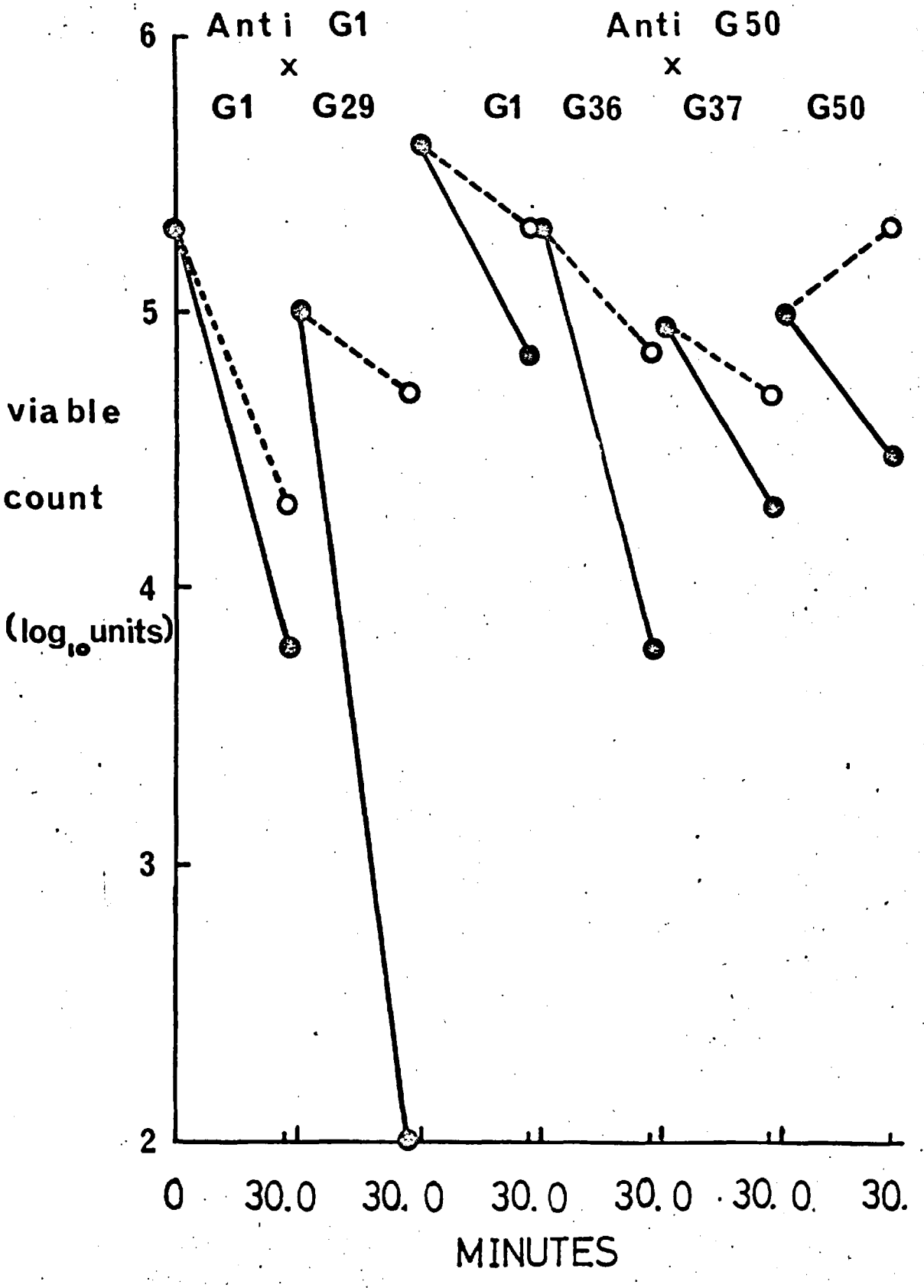
Reduction of bactericidal and haemagglutinating activity following absorption of immune sera swith lipopolysaccharide coated red cells.



Anti G1 absorbed with G1 lipopolysaccharide.

Anti G50 absorbed with G36 lipopolysaccharide.

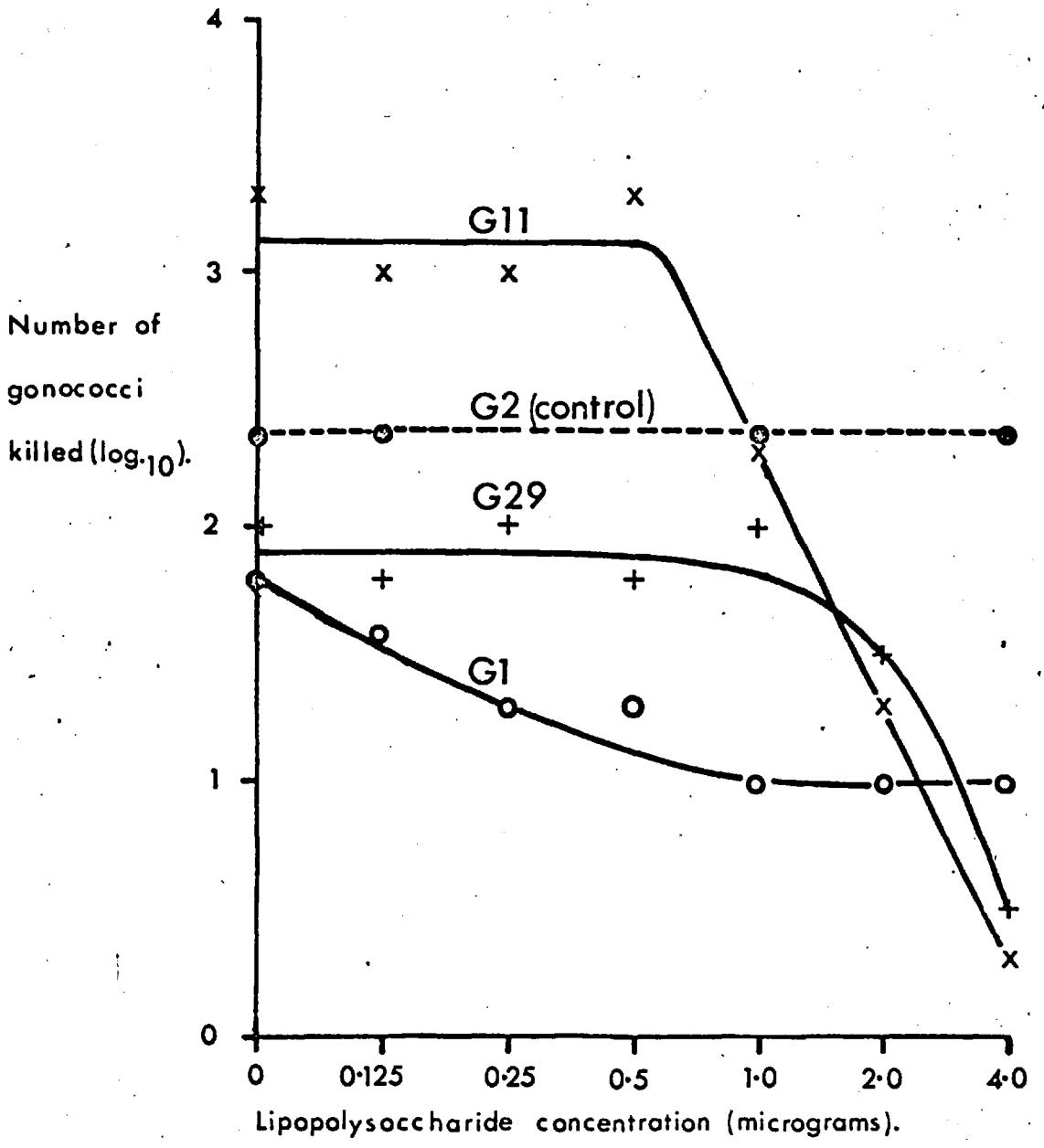
The haemagglutination titre of anti G1 was 10,240 before absorption and 4 after. The corresponding titres of anti G50 were 640 and 4.


Anti G1
x
G1 G29
Anti G50
x
G1 G36 G37 G50
6
5
4
3
2
viable
count
(log10 units)
0 30.0 30.0 30.0 30.0 30.0 30.
MINUTES

followed by 3 hr. at 4°C. The neutralised sera were then tested for their ability to provide antibody for the serum killing of strains G1, G11 and G29. The bactericidal test was done as previously (section 2.3.1.) with the exception that to minimise any possible anticomplementary effect due to the presence of antigen-antibody complexes 80% v/v of serum MW was used to provide a large excess of complement (section 4.2.4.). The results are shown in Fig. 8. Between 1-2µg of lipopolysaccharide in the experimental tubes was sufficient to measurably inhibit the serum killing of strains G11 or G29, whilst a concentration of 4µg totally abolished it. Killing of strain G1 by the homologous antiserum was measurably inhibited by as little as 0.125µg. However, the kinetics of this inhibition effect are clearly different, no further inhibition of serum killing occurring with concentrations of lipopolysaccharide in excess of 1µg/experimental tube. These results were not due to any anticomplementary effect, since the killing of strain G2 by serum MW was more than 2.3 log. units (>99%) in the presence of anti G1 serum which had been neutralised with different concentrations of lipopolysaccharide. These findings therefore support those of the preceding section where, using a different technique, it was found that absorption of anti G1 serum with G1 lipopolysaccharide only partially reduced its bactericidal activity for G1, but abolished it for G29. It seems certain, therefore, that gonococcal lipopolysaccharide is involved in the serum killing of subcultured gonococci.

Fig. 8.

Inhibitory effect of different concentrations of G1 lipopolysaccharide on the killing of N.gonorrhoeae strains G1, G11 and G29 by complement and anti G1 serum. The amount of lipopolysaccharide shown is the final concentration of the antigen in the experimental tubes. The anticomplementary control (dotted line) shows that the complement-mediated killing of strain G2, which is sensitive to normal human serum MW, was unaffected by the concentrations of lipopolysaccharide tested.


Number of gonococci killed (log_{10}).
4
3
2
1
0
G11
G2 (control)
G29
G1
0
0·125
0·25
0·5
1·0
2·0
4·0
Lipopolysaccharide concentration (micrograms).

C H A P T E R 5. Serological Properties of Gonococcal Lipopolysaccharide.

5.1.1. Introduction.

In the previous chapter it was shown that lipopolysaccharide is involved in the serum killing of gonococci. The group specificity of serum killing and the fact that many gonococci were resistant to killing by normal serum, suggested that lipopolysaccharide might form the basis of a serodiagnostic test for gonorrhoea or, alternatively, a method for distinguishing different strains of gonococci.

The acute and localised nature of gonococcal infections, particularly in the male, made it likely that any specific antibody response in the early stages of the disease would not be very marked. A sensitive technique for detecting antibody was therefore essential. The haemagglutination test was chosen because of the ease with which lipopolysaccharides may be attached to red cells (Davies et al., 1958) and the sensitivity of the method (Carpenter, 1965).

5.1.2. The choice of lipopolysaccharide for the haemagglutination test.

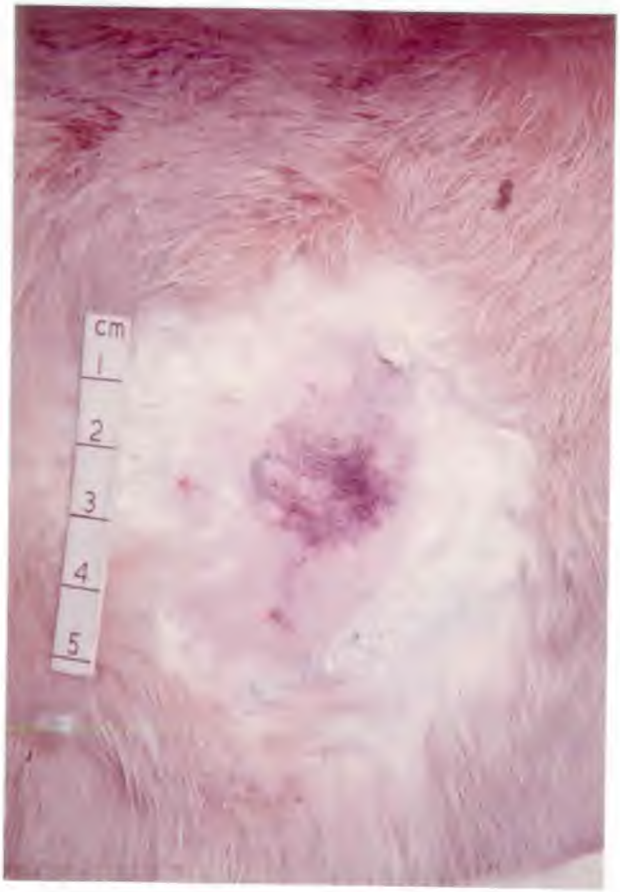
In an attempt to take account of the heterogeneity of gonococcal antigens and avoid the possibility of cross reactions with normal sera, lipopolysaccharides were prepared from 5 strains of gonococci. Four of these strains, G1, G22, G36 and G38, were resistant to normal serum in the bactericidal test, and represented provisional serogroups 2, 4, 3 and 4, respectively (see section 4.3.1.). The remaining strain, G2, was sensitive to normal serum (group 1) and was included for comparison.

Lipopolysaccharide was extracted by the phenol-water method (Westphal et al., 1952) as modified by Maeland (1968) and described in appendix 1. The yields of purified lipopolysaccharide obtained from strains G1, G2, G22, G36 and G38 were between 2-3 mg dry weight/gram of wet bacteria. Ultraviolet spectra of the purified lipopolysaccharides at a concentration of 0.1 mg/ml were very similar to those described by Maeland. The absence of absorption peaks at 260 and 280 nm indicated that the preparations were relatively free of contaminating nucleic acid or protein. 2.5µg of G1 lipopolysaccharide in pyrogen-free saline injected into a rabbit under the conditions of the British Pharmacoepia pyrogen test (BP 1969 App XV h) elicited a biphasic temperature response as measured by a rectal probe, together with an increase in the respiration rate within 3 minutes. This biphasic temperature response is considered to be highly characteristic of endotoxin (Milner & Finkelstein 1966). 75µg of G36 lipopolysaccharide injected intradermally into the shaved hind leg of a rabbit, followed 24 hours later by 10µg of the same preparation intravenously, produced a localised Shwartzman response (Fig. 9). 75µg or less of the same preparation injected intravenously was lethal for a rabbit within 12 hr., although in Maeland's experiments 100µg of similar preparations intravenously were tolerated (Maeland, 1968). The preparations therefore possessed endotoxic activity.

The minimal quantities of lipopolysaccharide required to sensitise optimally an equal volume of 2.5% v/v sheep red cells was 12.5, 6.25, 12.5, 12.5 and 12.5µg/ml for strains G1, G2, G22, G36 and G38 respectively. However, mild alkali treatment was necessary before the lipopolysaccharides

Fig. 9.

Localised Shwartzman response produced by gonococcal lipopolysaccharide. 75µg of G36 phenol-water extract was injected intradermally into the hind leg of a rabbit followed 24 hr. later by 10µg of the same preparation intravenously. The result confirms the endotoxic nature of the extract.


cm
1
2
3
4
5

would adhere to sheep red cells, in agreement with the finding of Maeland (1968).

5.2.1. Reactivity of gonococcal lipopolysaccharides with antisera to other bacterial species.

Tables 15 and 16 show the ability of antisera against a wide range of organisms to agglutinate red cells coated with various gonococcal lipopolysaccharides. Antisera to pneumococci, streptococci, salmonellae and human blood groups were chosen because the structure of the corresponding immunodeterminants is in most cases relatively well established. Any cross reactions between these antisera and gonococcal lipopolysaccharide might therefore conceivably give some indications as to the structure of the latter's immunodeterminants. Antisera to other Gram negative bacteria were chosen because they could be antigenically related to the Neisseria or be partially responsible for the development of natural antibodies bactericidal for gonococci (see section 4.4.2.). However, only antisera to meningococci, particularly to groups B and C, were capable of agglutinating gonococcal lipopolysaccharide coated red cells (Table 16). The absence of cross reactions with other organisms suggests that gonococcal lipopolysaccharides have a relatively unique structure and might therefore be sufficiently specific for the serodiagnosis of gonorrhoea.

5.2.2. Group Specificity of Gonococcal Lipopolysaccharide.

The ability of different gonococcal antisera to agglutinate red

TABLE 15

Antisera giving haemagglutinin titres of less than 40 with G1, G2 and G36 lipopolysaccharide coated red cells.

Pneumococcal antisera (Statens Serum Institut, Copenhagen).

Pneumococcus Pool	A	Types - 1, 2, 4, 5, 18.
	B	- 3, 6, 8, 19.
	C	- 7, 20, 24, 31, 40.
	D	- 9, 11, 16, 36, 37.
	E	- 10, 12, 21, 33, 39.
	F	- 17, 22, 27, 32, 41.
	G	- 29, 34, 35, 42, 47.
	H	- 13, 14, 15, 23, 28.
	I	- 25, 38, 43, 44, 45, 46, 48.

Streptococcal antisera (Wellcome Foundation and Dr. G. Coleman)

Streptococcus Lancefield Group	A	
	B	
	C	
	D	
	E	
	F	Otten's Type I, II, III, IV or V.
	G	
	N	

Salmonella antisera (Wellcome Foundation)

Salmonella Polyvalent 'O'.
'O' 5
'O' 4
'O' 3, 10, 15, 19.
'O' 6, 7.
'O' 13, 22.
'O' 8
'O' 2
'O' 9

Salmonella Vi

TABLE 15 (cont'd)

Escherichia Coli antisera (Wellcome Foundation)

E. coli	O	125	B15
	O	111	B4
	O	119	B14
	O	26	B6
	O	126	B16
	O	128	B12
Polyvalent	O	Pool 2	
Polyvalent	O	Pool 3	

Antisera to other Gram negative organisms (Wellcome Foundation)

V. cholerae Polyvalent.

Shigella boydii	1 - 6
	7 - 11
	12 - 15
Shigella dysenteriae	1, 2.
Shigella sonnei	1, 2.
Shigella flexneri Polyvalent	1-6.

Human blood group sera (Blood Group Reference Laboratory)

Group A

Group B

Ulex europaeusextract (A.A. Glynn). Agglutinates red cells which, like human group O cells, contain group H substance.

TABLE 16

Antisera giving positive haemagglutinin titres to G1, G2, G22, G36 or G38 lipopolysaccharide coated red cells.

Antisera to	Haemagglutinin titres to sensitising lipopolysaccharides from strains G1	G2	G22	G36	G38
Neisseria meningitidis Group A	<40	160	<40	<40	<40
Group B	640	640	640	320	160
Group C	320	320	320	160	<40
Group D	<40	320	<40	<40	<40
Neisseria gonorrhoeae G1	1280				
G2		1280			
G22			2560		
G37				640	
G38					640

cells coated with gonococcal lipopolysaccharide is shown in Table 17. Although all the cells were sensitised by the same technique, comparison is made difficult because they probably differed in agglutinability, whilst the antisera must also have differed in their potency. Nevertheless, wherever it was possible to test a serum against its homologous lipopolysaccharide the haemagglutination titre was satisfactory. Therefore, even allowing for variations due to these factors it is clear that gonococcal lipopolysaccharide showed some degree of group specificity. Thus G2 lipopolysaccharide can be distinguished from G36 lipopolysaccharide because the latter was not agglutinated by anti G38 or anti G22. Similarly, G1 lipopolysaccharide can be distinguished from G2 lipopolysaccharide since the former was not agglutinated by anti G3, anti G4, anti G37, anti G38 or anti G50.

5.2.3. Serological specificity of Thomas and Mennie's extract.

The practicability of the haemagglutination test as a method for distinguishing between gonococcal strains is limited by the tediousness of the lipopolysaccharide extraction. As reviewed in section 1.2.3. Chanarin (1954) using a simple extraction procedure advocated by Thomas and Mennie (1950) involving digestion of gonococci with 0.05 N sodium hydroxide obtained a group specific polysaccharide red cell-sensitising extract. In view of the extraction procedure and its ready adherance to red cells, it seemed possible this material might represent alkali degraded lipopolysaccharide. The specificity of Thomas and Mennie's extract obtained from strains G1, G2 and G36, was therefore compared

TABLE 17

Haemagglutination titres of immune anti-gonococcal sera tested against sheep red cells sensitised with gonococcal lipopolysaccharides.

Sensitising lipopoly-saccharide from strain	Haemagglutinin titres of										
	Antisera to strain										Pooled N.H.S.
	G1	G2	G3	G4	G7	G22	G37	G38	G50	G52	
G1	1280	640	40	40	320	80	40	<40	40	640	16
G2	2560	1280	1280	1280	5120	2560	2560	2560	640	5120	16
G22	1280	<40	640	80	1280	2560	80	2560	1280	1280	16
G36	40	40	40	320	80	<40	320	<40	80	<40	8
G38	320	<40	640	160	640	320	160	640	80	640	32

with the corresponding lipopolysaccharides in the haemagglutination test. However, it is clear from the results in Table 18 that the specificity of the Thomas and Mennie extract is much broader than that of the corresponding lipopolysaccharides. Thus red cells coated with a Thomas and Mennie's extract of strain G1 were agglutinated to a much higher titre by anti G37 serum than were red cells coated with the purified lipopolysaccharide from the same strain. This extract was therefore less satisfactory for the serotyping of gonococci.

Attempts to make the extraction of gonococcal lipopolysaccharide less laborious by doing it on a semi-micro scale and omitting the ultracentrifugation failed because the resultant crude preparations would not sensitise red cells. However, the preparation did not inhibit the agglutination of sheep erythrocytes by Forssman antibody (Glynn and Howard, 1970) suggesting that antigens analogous to the K antigens of E.coli were not responsible for inhibiting sensitisation.

5.2.4. Comparison of the results of the haemagglutination and bactericidal tests.

In Table 19 the results of the haemagglutination tests are compared where possible with the sensitivity of the corresponding strain to the same sera in the bactericidal test. Strain G1 was killed by anti G1 and anti G2, doubtfully by anti G3 and anti G4, and not at all by anti G37 and anti G50. The haemagglutinin titres corresponded.

G2 was killed by many normal human sera and its lipopolysaccharide reacted with all the immune sera. Furthermore G2 lipopolysaccharide was

TABLE 18

Serological specificity of Thomas and Mennies extract compared with the phenol water lipopolysaccharide from the same gonococcal strains.

Antisera to strain	Agglutination titres of red cells sensitised with:			
	Phenol-water lipopolysaccharide		Thomas and Mennies extract	
	from strains: G1	G36	G1	G36
G1	1280	40	640	640
G2	640	40	640	640
G37	40	640	2560	2560
G50	40	160	640	640

TABLE 19

Comparison of the haemagglutination and bactericidal tests.

Serum	1) Haemagglutination titre, or 2) sensitivity to serum killing[b] of strains:									
	G1		G2		G22		G36		G38	
	1	2	1	2	1	2	1	2	1	2
NHS[a]	16	-	16	++	16	-	8	-	32	-
anti G1	1280	++	2560		1280	-	40	-	320	-
" G2	640	++	1280		<40	-	40	-	<40	-
" G3	40	+	1280		640		40	-	640	-
" G4	40	+	1280		80	-	320	-	160	-
" G7	320	-	5120		1280	-	80		640	-
" G22	80	-	2560		2560	-	<40		320	-
" G37	40	-	2560		80	-	320	++	160	-
" G38	<40	-	2560		2560	-	<40		640	++
" G50	40	-	640		1280	-	80	++	80	-
" G52	640	-	5120		1280	-	<40		640	-

a) NHS = Normal Human Serum.

b) Sensitivity to serum killing:

++ = $\geq$ 90% killing.

+ = $>$ 50 $\leq$ 89% killing.

- = $\leq$ 50% killing.

the only one of the five tested to be agglutinated by antisera to meningococci of groups A, B, C and D. It is therefore possible that G2 represents a rough strain in which some of the group specific immunodeterminants have been lost.

Strain G36 was killed by anti G37 and anti G50, but not by anti G4. However, its lipopolysaccharide reacted with all three sera.

Strain G38 was only killed by the homologous antiserum, but like G22 which was killed by none of the antisera, its lipopolysaccharide reacted with many.

Whenever tested therefore, antisera killing a given strain always reacted with the corresponding lipopolysaccharide. However, a positive haemagglutination result was not always followed by killing. The implications of this finding are discussed in chapter 8.

5.2.5. Intra-Neisserial specificity of G1 lipopolysaccharide.

The ability of anti G1 serum absorbed with various species of Neisseria to agglutinate G1 lipopolysaccharide coated red cells is shown in Table 20. For comparison the ability of the identical sera to kill G1 in the bactericidal test is also shown.

None of the strains tested, except gonococci, were capable of absorbing haemagglutinins from anti G1 serum, demonstrating that they did not share antigenic determinants with G1 lipopolysaccharide. This result is surprising since it is known that meningococci and gonococci share common antigenic determinants (see section 1.2.3.).

Bactericidal activity for the homologous strain was repeatedly

TABLE 20

Intra Neisserial specificity of absorbed anti G1 serum in the haemagglutination and bactericidal tests.

Antiserum to strain	Absorbed with	Haemagglutination and serum killing of strain G1	
		Haemagglutination titre	Sensitivity to serum killing [a]
G1	Unabsorbed	640	++
	N.gonorrhoeae G1 100°C 1 hr	<40	-
	N.gonorrhoeae G1 121°C 2 hr	<40	-
	N.gonorrhoeae G2	40	-
	N.gonorrhoeae G29	<40	-
	N.gonorrhoeae G36	40	++
	N.meningitidis NCTC 8339	320	++
	N.pharyngis-siccus NCTC 4591	640	-
	N.pharyngis-flavus NCTC 4590	640	++
	N.catarrhalis NCTC 3622	640	++
	N.flavescens NCTC 8263	640	++
G2	Unabsorbed	640	++
	N.gonorrhoeae G2 100°C 1 hr	<40	-
	N.gonorrhoeae G36	320	++

a = Sensitivity to serum killing (serum MW).

++ = 90% killing.
+ = 50 89% killing.
- = 50% killing.

removed from anti G1 serum by absorption with N.pharyngis siccus NCTC 4591. However, this did not affect the ability of the identical serum to agglutinate G1 lipopolysaccharide coated erythrocytes. A possible explanation of this result is discussed in chapter 8.

5.2.6. Specific inhibition of the haemagglutination reaction with simple sugars.

Maeland (1969b) demonstrated that the haemagglutination of lipopolysaccharide coated red cells with antisera could be inhibited by certain sugars, suggesting that they were involved in the structure of the immunodeterminant. This work was limited, however, in that lipopolysaccharide was only prepared from one gonococcal strain. It was therefore not clear whether the postulated immunodeterminant structure was group or species specific; nor whether other sugars might inhibit the reactivity of different lipopolysaccharides.

Sugar inhibition tests were performed as described by Maeland (1969b). 0.1 ml volumes of 0.56 M sugar solutions were incubated for 1 hr. at 37°C, and then overnight at 4°C with 0.1 ml volumes of doubling dilutions of antisera. 0.1 ml of the appropriate lipopolysaccharide on glutaraldehyde fixed red cells were then added. The haemagglutination was then performed as described in section 2.4.4.

The agglutination of G36 coated red cells was inhibited by final concentrations of 0.28 M galactose or, more markedly, by 0.28 M α lactose (Table 21). However, agglutination of none of the other sensitised red cells was inhibited by the sugars tested.

TABLE 21

Effect of neutralisation with sugars on the haemagglutination titre of antibodies to gonococcal lipopolysaccharides.

Sensitising lipopolysaccharide from strain:	Antiserum to strain:	Titre of unabsorbed serum	Haemagglutination titres of antisera absorbed with the following 0.28 M[a] sugars:										
			D(+) Glucose	D(-) Fructose	D(+) Glucosamine	Sucrose	Maltose	Lactose	D(+) Galactose	D(+) Mannose	L(+) Rhamnose	L(-) Fucose	L(+) Arabinose
G1	G1	1280	1280	2560	2560	2560	1280	1280	2560	2560	2560	2560	2560
G2	G2	2560	2560	2560	2560	2560	2560	2560	2560	2560	2560	2560	2560
G36	G37	640	640	640	640	640	640	80	160	640	640	640	640

a = final concentration.

To establish the minimal amount of sugars required to inhibit the agglutination of G36 lipopolysaccharide coated erythrocytes, the same procedure as before was followed but this time the concentrations of galactose and α or β lactose were varied. As Table 22 shows, a final concentration of at least 0.07 M α or β lactose, or 0.14 M galactose, was required to inhibit this agglutination by at least two dilution steps.

Detection of gonococcal antibodies.

5.3.1. The antibody response to gonococcal lipopolysaccharides.

The potential usefulness of a serodiagnostic test for gonorrhoea, particularly in asymptomatic gonococcal carriers, has been outlined in chapter 1. The possibility of using the haemagglutination test for this purpose was therefore investigated.

Consecutive sera were obtained from 50 male and 25 female patients attending the venereal diseases department of St. Mary's Hospital with positive smears for Neisseria. Control normal sera were also obtained from 50 donors of either sex submitting blood for grouping. After triple absorption with 10% v/v of packed sheep red cells for 18-20 hours at $4^{\circ}C$ to remove Forssman antibody, the sera at a 1:2 dilution no longer agglutinated sheep red cells.

The ability of these sera to agglutinate glutaraldehyde fixed sheep cells coated with G1, G2 or G36 lipopolysaccharides is shown in Fig. 10, together with the geometric mean haemagglutination titre and the 95% confidence limits for the population mean. Taking as end points

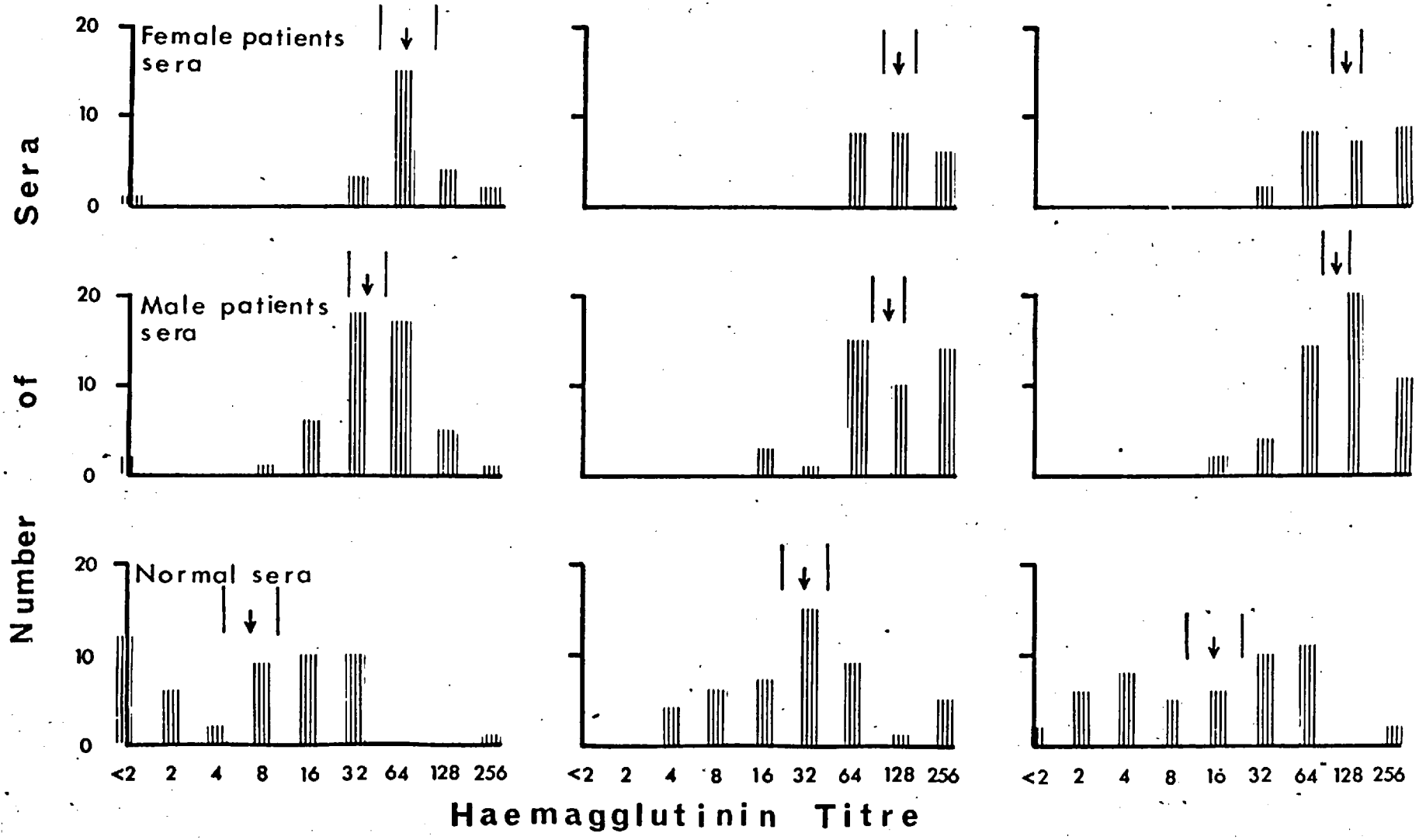
TABLE 22

Concentration of various sugars required to inhibit the agglutination of G36 lipopolysaccharide coated red cells by anti G37.

Absorbing sugar	Final concentration	Haemagglutination titre
None		640
D(+) Galactose	0.035 M	640
	0.07 M	640
	0.14 M	160
	0.28 M	160
alpha Lactose	0.035 M	320
	0.07 M	160
	0.14 M	80
	0.28 M	80
beta Lactose	0.035 M	640
	0.07 M	160
	0.14 M	80
	0.28 M	40
alpha D Melibiose	0.035 M	640
	0.07 M	640
	0.14 M	640
	0.28 M	640

Fig. 10.

Agglutination of gonococcal lipopolysaccharide coated red cells by normal and patients' sera. The vertical arrow and two parallel lines show the sample geometric mean titre with the upper and lower 95% confidence limits for the corresponding population mean.


G1 lipopolysaccharide
G2 lipopolysaccharide
G36 lipolysaccharide
Female patients sera
Male patients sera
Normal sera
Number of Sera
20
10
0
<2 2 4 8 16 32 64 128 256
Haemagglutinin Titre

haemagglutinin titres of greater than 16, 32 and 32 for G1, G2 and G36 lipopolysaccharides respectively, cross reacting antibody could be correspondingly detected in 87, 94 and 89% of male and female patients' sera compared with 22, 32 and 26% of control normal sera. To confirm that there is a significant difference between patients' or normal sera in their ability to agglutinate red cells coated with a particular antigen the sample means were compared using a t test for unpaired data. The sample variances were also compared, using an F test (Table 23). In all cases, sera from male or female patients reacted with gonococcal lipopolysaccharides to a significantly higher geometric mean titre than the control sera, the probability of the results being due to chance being less than 0.1%. However, there was little or no significant difference between the geometric mean titres of male or female patients' sera obtained with each of the three different lipopolysaccharides. Because the degree of sensitisation probably differed for each of the three lipopolysaccharide preparations, it was not possible to test whether they showed any significant differences in their ability to detect gonococcal antibody.

Comparison of the sample variances using an F test showed, as expected from Fig. 10, that the dispersion of titres using normal sera was, in general, significantly greater than with patients' sera. However, there was little or no significant difference in the sample variances when male and female sera were compared. This may be explained by the fact that, as suggested by the bactericidal experiments in section 4.4.2., cross reacting normal antibody has probably arisen as a result of highly

TABLE 23.

Statistical analysis of data in Fig. 10.

1) G1 Lipopolysaccharide

Sera tested	Geometric mean titre $\bar{x}$ and $\bar{y}$	Variance S^2_x, S^2_y	t test, $\bar{x} > \bar{y}$			F test for sample variances			
			d.f	t	P	Test	d.f	F	P
Male patients, x	5.240	2.104	98	7.195	<0.1% H.sig.[a]	$S^2_x < S^2_y$	49	1.98	> 0.1% < 1.0% H.sig.
Normal, y	2.700	4.173					49		
Female patients, x	5.960	2.123	73	7.133	< 0.1% H.sig.	$S^2_x < S^2_y$	24	1.96	> 1.0% < 5.0% Sig.
Normal, y	2.700	4.173					49		
Female patients, x	5.960	2.123	73	2.033	> 1.25% < 2.5% Sig.	$S^2_x > S^2_y$	24	1.01	> 5.0% Not sig.
Male patients, y	5.240	2.104					49		

a = Significance. H.sig. = Highly significant. Sig. = Probably significant. Not sig. = not significant.

TABLE 23. (cont'd) Statistical analysis of data in Fig. 10.

2) G2 Lipopolysaccharide

Sera tested	Geometric mean titre $\bar{x}$ and $\bar{y}$	Variance S^2_x, S^2_y	t test, $\bar{x} > \bar{y}$			F test for sample variances			
			d.f	t	P	Test	d.f	F	P
Male patients, x	6.720	1.348	88	6.090	<0.1% H.sig.	$S^2_x < S^2_y$	42	2.01	>0.1%<1.0% H.sig.
Normal, y	4.893	2.705					46		
Female patients, x	6.909	0.658	67	5.478	<0.1% H.sig.	$S^2_x < S^2_y$	21	4.11	<0.1% H.sig.
Normal, y	4.893	2.705					46		
Female patients, x	6.909	0.658	63	0.687	>25% Not sig	$S^2_x < S^2_y$	21	2.05	>1.0%<5.0% Sig.
Male patients, y	6.720	1.348					42		

TABLE 23. (cont'd) Statistical analysis of data in Fig. 10.

3) G36 Lipopolysaccharide.

Sera tested	Geometric mean titre $\bar{x}$ and $\bar{y}$	Variance S^2_x, S^2_y	t test, $\bar{x} > \bar{y}$			F test for sample variances			
			d.f	t	P	Test	d.f	F	P
Male patients, x	6.640	1.051	98	8.580	< 0.1% H.sig.	$S^2_x < S^2_y$	49	4.04	< 0.1% H.sig.
Normal, y	3.860	4.245					49		
Female patients, x	6.840	0.973	73	6.850	< 0.1% H.sig.	$S^2_x < S^2_y$	24	4.36	< 0.1% H.sig.
Normal, y	3.860	4.245					49		
Female patients, x	6.840	0.973	73	0.813	> 20.0% Not sig.	$S^2_x < S^2_y$	24	1.08	> 5.0% Not sig.
Male patients, y	6.640	1.051					49		

variable antigenic stimulation by a wide range of organisms. Nevertheless this is unlikely to be the sole explanation since the variance of titres obtained with the normal sera using lipopolysaccharide derived from strain G2, which in the bactericidal and haemagglutination tests reacts with a wide range of antibodies (see sections 4.3.1. and 5.2.2.) was less than that obtained from the serologically more specific strains G1 and G36. The finding that there is little significant difference in the sample variances when male and female patients' sera are compared is surprising, in that there was a considerably greater degree of variation in the length of gonococcal infection amongst females than males. This could tentatively mean that in females gonococcal antibody is produced relatively early following infection. Table 24 relates the duration of urethral discharge amongst 50 male patients with gonorrhoea to the geometric mean agglutination titre obtained using the three different lipopolysaccharide preparations. Although the number of sera in some of the groups is too small for adequate statistical analysis, there was surprisingly little difference between them. Again this may mean that the antibody response follows soon after the infection.

If the haemagglutination titres of the same sera using different lipopolysaccharide antigens are related, then when the titres obtained with different pairs of lipopolysaccharide are compared in a scatter diagram, the points should fall on or near a regression line. In Fig. 11 the regression line through the origin for each of the pairs of data has been plotted. The 95% confidence limits are also indicated to give a visual indication of the standard error about the regression line. In

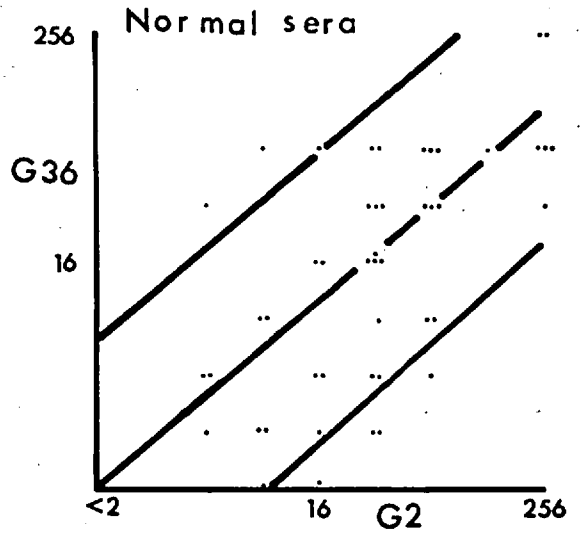
TABLE 24

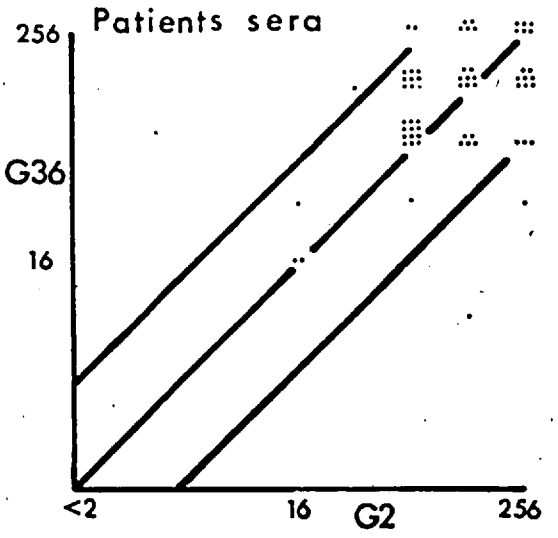
Geometric mean haemagglutination titre of 50 male patients' sera for G1, G2 and G36 lipopolysaccharide coated red cells as a function of the duration of infection.

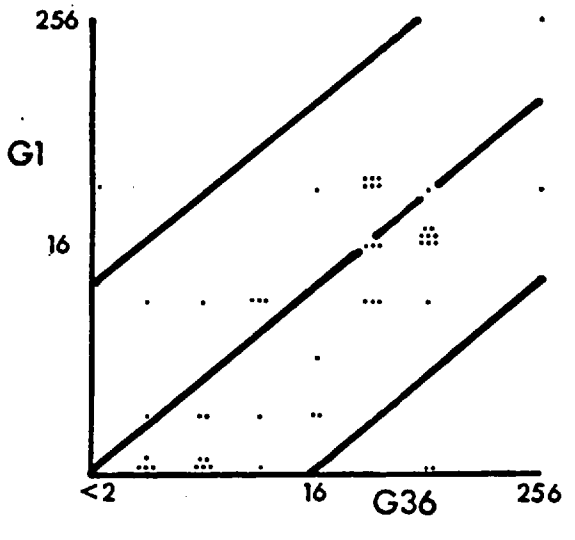
Duration of Discharge (Days)	Number of Sera			Geometric Mean Titre		
	G1	G2	G36	G1	G2	G36
1 - 3	26	24	26	5.0	6.9	6.6
4 - 5	6	6	6	5.5	7.0	5.8
6 - 10	1	1	1	5.0	6.0	8.0
>10	4	2	4	5.8	7.0	7.0
Multiple Infections	13	10	13	5.8	6.9	6.9

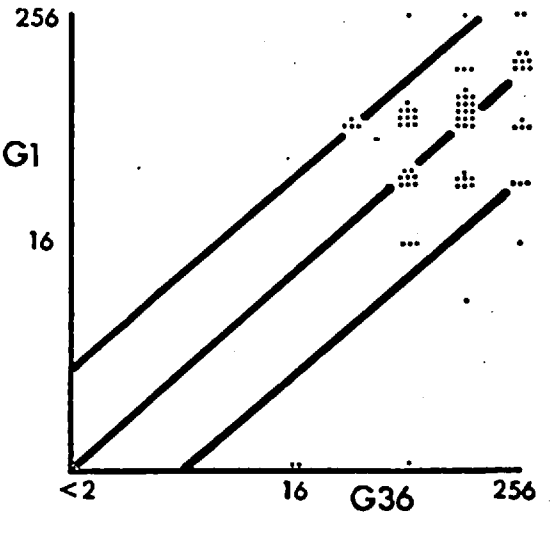
Fig. 11.

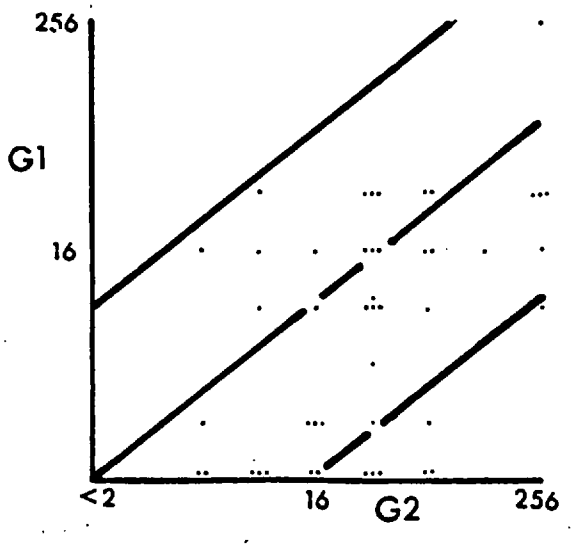
Comparison of the haemagglutination titres obtained for the same sera using different lipopolysaccharides. The parallel lines are the regression line through the origin together with its upper and lower 95% confidence limits.

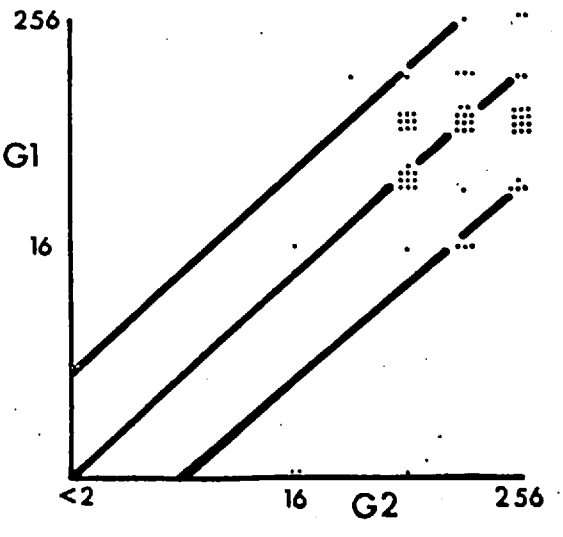

Normal sera
256
G36
16
<2
16
G2
256


Patients sera
256
G36
16
<2
16
G2
256


256
G1
16
<2
16
G36
256


256
G1
16
<2
16
G36
256


256
G1
16
<2
16
G2
256


256
G1
16
<2
16
G2
256

general, as the width of the confidence limits suggest, patients' sera more frequently gave similar titres with all three lipopolysaccharide preparations than did the normal sera. Again this is probably because the natural antibodies reacting with gonococcal lipopolysaccharide have been produced as the result of a more varied antigenic stimulus than the antibodies in the patients' sera.

The data presented in this chapter strongly suggests that there is an early antibody response to gonococcal lipopolysaccharide in male and female patients with gonorrhoea. However, between 70-100% of the titres for the patients' sera fell within + 1.96 standard deviations of the geometric mean titre for the normal sera. Further work is therefore in progress, now that it is clear that antibodies are produced to the lipopolysaccharide, to modify the detection system so that it can be used for the diagnosis of gonorrhoea. The finding that gonococcal lipopolysaccharides possess group or type specific immunodeterminants, which has been independently supported by the work of Maeland (1969d), suggests that lipopolysaccharide may also form the basis of a serological typing system for gonococci which would be useful in epidemiological studies.

CHAPTER 6. A virulence attribute of gonococci lost on subculture.

6.1.1. Introduction.

One of the most important attributes of a virulent organism is that it must be relatively resistant to the host's defence mechanisms. However, nobody has yet reported any significant differences in this respect between virulent and avirulent gonococci which could be causally related to their pathogenicity (Kellogg, 1963, 1968). Since gonorrhoea currently involves some 100 million patients per year (Catterall, 1969) and repeat infections frequently occur, there can be little natural resistance to the disease and nor can significant immunity be acquired as a result of the infection. This is supported by the observations of Holmes et al. (1970) who found that amongst the crew of an American aircraft carrier about 50% of those admitting sexual intercourse with females having culturally proven gonorrhoea developed the disease. However, it has been known for a long time that normal human serum is bactericidal for subcultured gonococci (Spink and Keefer, 1937) and it seems probable that they may also be destroyed by human polymorphs (Watt, 1970, To be published). How then are gonococci able to persist despite the host's inflammatory response?

One possible explanation might be that since the natural bactericidal antibodies are strain specific (see section 4.4.1.) gonococci only infect individuals lacking antibody to the strain concerned. Thus Goldschneider et al. (1969) showed that army recruits lacking group specific natural bactericidal antibody to meningococci were more likely than those

possessing it subsequently to develop meningococcal meningitis. Nevertheless it is not feasible to explain the high incidence and rate of infection found in gonorrhoea solely on the basis of host defence deficiencies.

An alternative possibility is that gonococci growing in the host are resistant to serum killing and possibly to destruction by polymorphs. Certainly the enormous doses of cultured gonococci required to infect volunteers (Kellogg et al., 1963; Kellog and Thayer, 1969; WHO 1965, Mahoney et al., 1946) suggests that virulence attributes are rapidly lost on subculture.

To distinguish between these possibilities the resistance of gonococci present in exudate and the same organism after subculture to killing by the homologous patients' serum has been compared.

6.1.2. The recovery of gonococci from urethral exudate.

Urethral exudates and serum were collected from men with acute gonorrhoea of between 1 to 3 days duration only. This thereby lessened the possibility that antibodies acquired as a result of the infection might mask any deficiencies in the ability of the patients' natural antibodies to kill the invading organism. After staining to confirm the presence of Gram negative diplococci the exudates were suspended in 0.5 ml of Dulbecco's solution and maintained at 37°C. At the same time blood was collected from the patient and the serum separated as described in section 2.2.1. so as to preserve the complement. A portion of this serum sufficient for the bactericidal test was kept on flake ice, and the remainder was deep frozen at -70°C for further use. Just prior to the experiment the exudate suspension was centrifuged and the supernatant

retained for analysis by immunodiffusion (see section 6.3.1.). The precipitate was then resuspended in Dulbecco's solution and ground for approximately 20 seconds in a Teflon grinder (Pierce et al., 1953). This procedure ruptured the polymorphs and cell debris present, but did not significantly affect the viability of the exudate gonococci. The organisms were then washed twice and resuspended in Dulbecco's solution to remove soluble contaminants.

The bactericidal test was done by incubating 0.1 ml of the exudate gonococci suspension with 0.4 ml of the homologous fresh human serum. The final concentration of serum, 80%, was higher than used previously so that any slight bactericidal activity due to traces of natural antibody would not be affected by dilution. Viable counts were performed at the start of the experiment on the inocolum in Dulbecco's solution, and again on the gonococci surviving in the experimental tubes after incubation for 1 hr. at 37°C. The serum sensitivity of the subcultured gonococci was tested in the same way. However, between two and three passages on Amies and Garabedian medium were generally necessary in order to purify the organism and to provide sufficient material for preservation by lyophilisation. Before testing each of the subcultured gonococci was standardised to the logarithmic growth phase using the modified biphasic flask (section 2.1.5.).

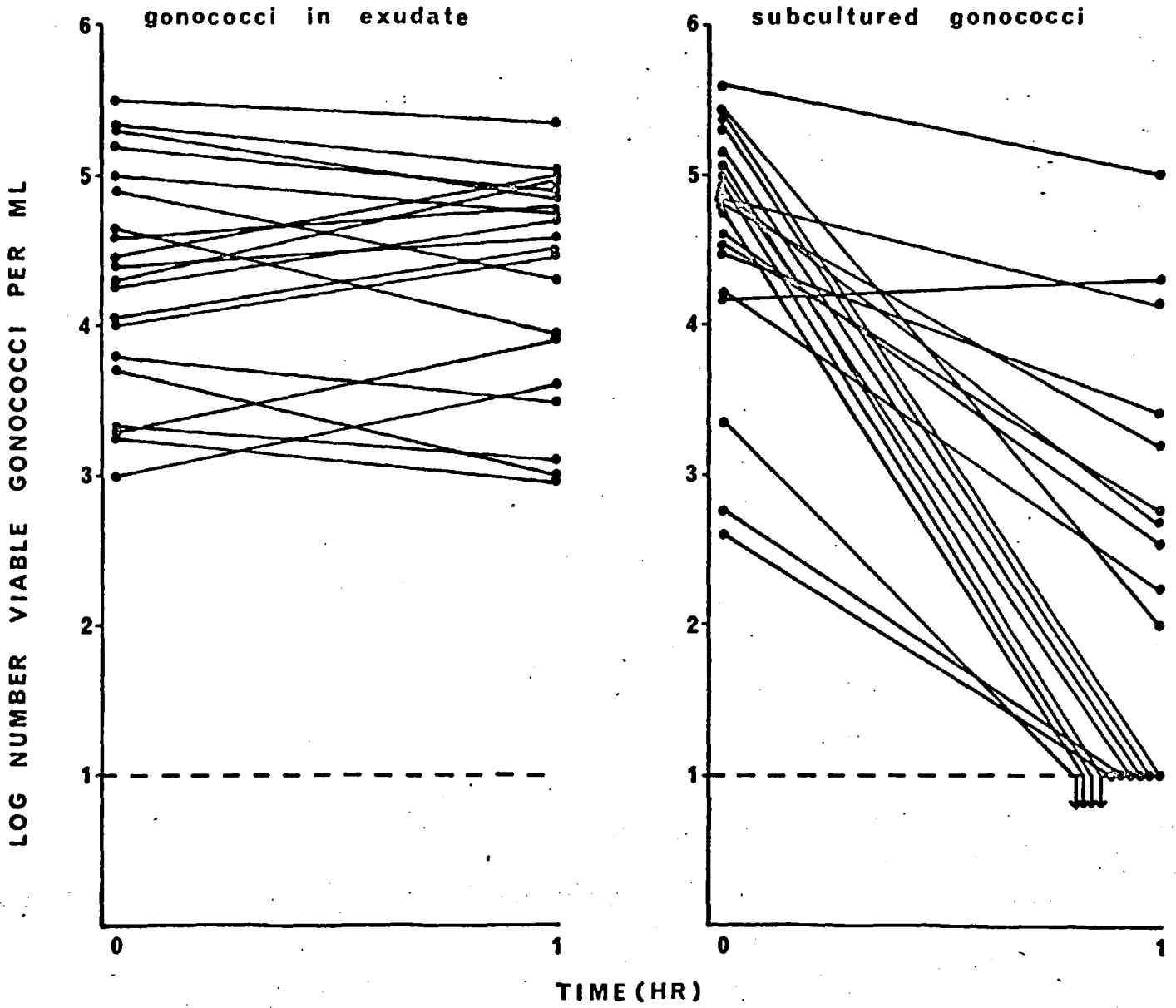
Comparison between host grown gonococci and the same strain after subculture.

6.2.1. The effect of passage in vitro on the susceptibility of exudate and subcultured gonococci to serum killing.

Fig. 12 visualises the results of 20 experiments on the susceptibility

Fig. 12.

The effect of patients' serum on the corresponding strains of N.gonorrhoeae both in the native exudates and after subculture. In this and Figs 13 and 14 each line represents an individual experiment so that the group of lines in each graph gives an indication of the scatter of the results.


gonococci in exudate
subcultured gonococci
LOG NUMBER VIABLE GONOCOCCI PER ML
6
5
4
3
2
1
0
1
TIME (HR)

of gonococci grown in the host, and the same strain after subculture, to killing by the homologous patients' serum. The results for each individual strain are shown in Table 25. The log mean number of exudate gonococci added to the serum was 4.3, and the average number recovered after one hour's incubation was again 4.3. Minor variations in individual experiments (e.g. G70, G83) are probably explained by the tendency of gonococci in exudate to form clumps. By contrast, 17 out of 20 of the same strains after subculture showed a reduction of 1 log unit (i.e. 90%) or more in viable count after incubation for 1 hr. in the corresponding patients' serum. Therefore most patients possess bactericidal antibodies capable of killing the infecting strain grown in vitro. However, these same antibodies failed to kill the gonococci present in urethral exudate even when, as with G74, the number of viable organisms was as low as 5×10^3.

It seems unlikely that the antibodies in the patients' sera killing subcultured gonococci were acquired as a result of the infection. This is because, as shown in Table 25, similar results were obtained using a normal human serum (MW). Exudate gonococci were again much more resistant to serum killing, the log mean number of gonococci added to the serum being 4.3, and the log mean number recovered after one hour's incubation was 4.1. In general those subcultured strains which were killed by the homologous patients' serum were also killed by serum MW.

6.2.2. The susceptibility of exudate gonococci to killing by human polymorphs.

One of the most obvious host responses to gonococcal infection is

TABLE 25

Survival of exudate and freshly subcultured gonococci after incubation for 60 min. in a normal serum (MW) and the homologous patients' sera.

Strain Number	Log_{10} Viable gonococci					
	In Exudate			After subculture		
	Inoculum	Patients serum	Normal serum	Inoculum	Patients serum	Normal serum
G70	4.3	5.0	5.0	4.8	3.2	3.3
G71	4.3	4.7	5.0	4.9	2.7	2.2
G72	4.9	4.3	3.7	4.2	2.3	2.2
G73	4.7	4.0	3.0	5.0	<1.0	<1.0
G74	3.3	4.0	3.3	3.4	<1.0	1.0
G77	3.0	3.6	3.8	2.8	1.0	2.7
G78	4.0	4.5	4.0	2.6	1.0	2.5
G79	5.3	4.9	4.5	4.6	2.5	2.3
G81	4.5	5.0	5.3	5.0	<1.0	<1.0
G83	3.7	3.0	3.9	5.4	<1.0	<1.0
G85	5.0	4.8	4.7	5.5	2.0	1.9
G87	3.3	3.0	2.3	5.6	5.0	5.3
G88	5.2	4.9	5.2	5.0	1.0	1.8
G89	4.4	4.6	4.3	4.5	3.4	3.7
G90	5.5	5.3	5.0	5.0	4.3	4.3
G100	4.6	4.5	4.3	5.3	<1.0	1.0
G101	5.3	5.0	4.6	4.8	<1.0	<1.0
G102	4.7	4.8	4.2	4.9	4.2	2.5
G103	3.9	3.6	3.2	5.2	1.0	1.9
G104	3.3	3.2	2.9	4.5	2.8	3.3
MEAN	4.3	4.3	4.1	4.6	1.8	2.0

the migration of polymorphonuclear leukocytes to the infected area. It therefore seemed relevant to investigate whether exudate gonococci were also resistant to destruction by human polymorphs.

Approximately 5 ml of blood from a donor free of previous gonococcal infection was treated with 0.03 mg. heparin/ml, centrifuged lightly and the polymorphs collected from the buffy coat. The majority of the polymorphs so obtained were healthy since they did not stain with Trypan Blue and were actively mobile when examined at 37°C under the phase contrast microscope. The polymorphs were suspended in fresh patients' serum and 0.4 ml of the mixture incubated as before with 0.1 ml of some of the strains of exudate gonococci previously described (section 6.2.1.).

Table 26 shows that the log mean number of exudate gonococci surviving in patients' serum **plus** polymorphs was virtually the same as the number surviving in patients' serum alone. However, examination of stained smears showed that little phagocytosis had occurred. It therefore seems probable that the failure of polymorphs to phagocytose exudate gonococci is an artifact of the in vitro test system since in the natural infection smears of the urethral exudate usually show that a large number of the gonococci present have been phagocytosed. The question as to whether polymorphs can kill host grown gonococci therefore remains, for the time being, unresolved.

6.2.3. The sensitivity of exudate and subcultured gonococci to a pooled antigonococcal serum.

It seemed possible that the resistance of exudate gonococci to serum

TABLE 26

The survival of exudate gonococci after incubation for 60 min. with patients' serum and polymorphs.

Strain Number	Log_{10} Viable gonococci		
	Inoculum	Patients' serum	Patients' serum & polymorphs
G70	4.3	5.0	5.0
G71	4.3	4.7	5.3
G72	4.9	4.3	4.3
G73	4.7	4.0	3.8
G74	3.3	4.0	3.5
G77	3.0	3.6	3.7
G78	4.0	4.5	4.3
G79	5.3	4.9	4.7
G81	4.5	5.0	5.0
G83	3.7	3.0	3.0
G85	5.0	4.8	4.3
G87	3.3	3.0	3.3
G88	5.2	4.9	4.5
G89	4.4	4.6	4.3
MEAN:	4.3	4.3	4.2

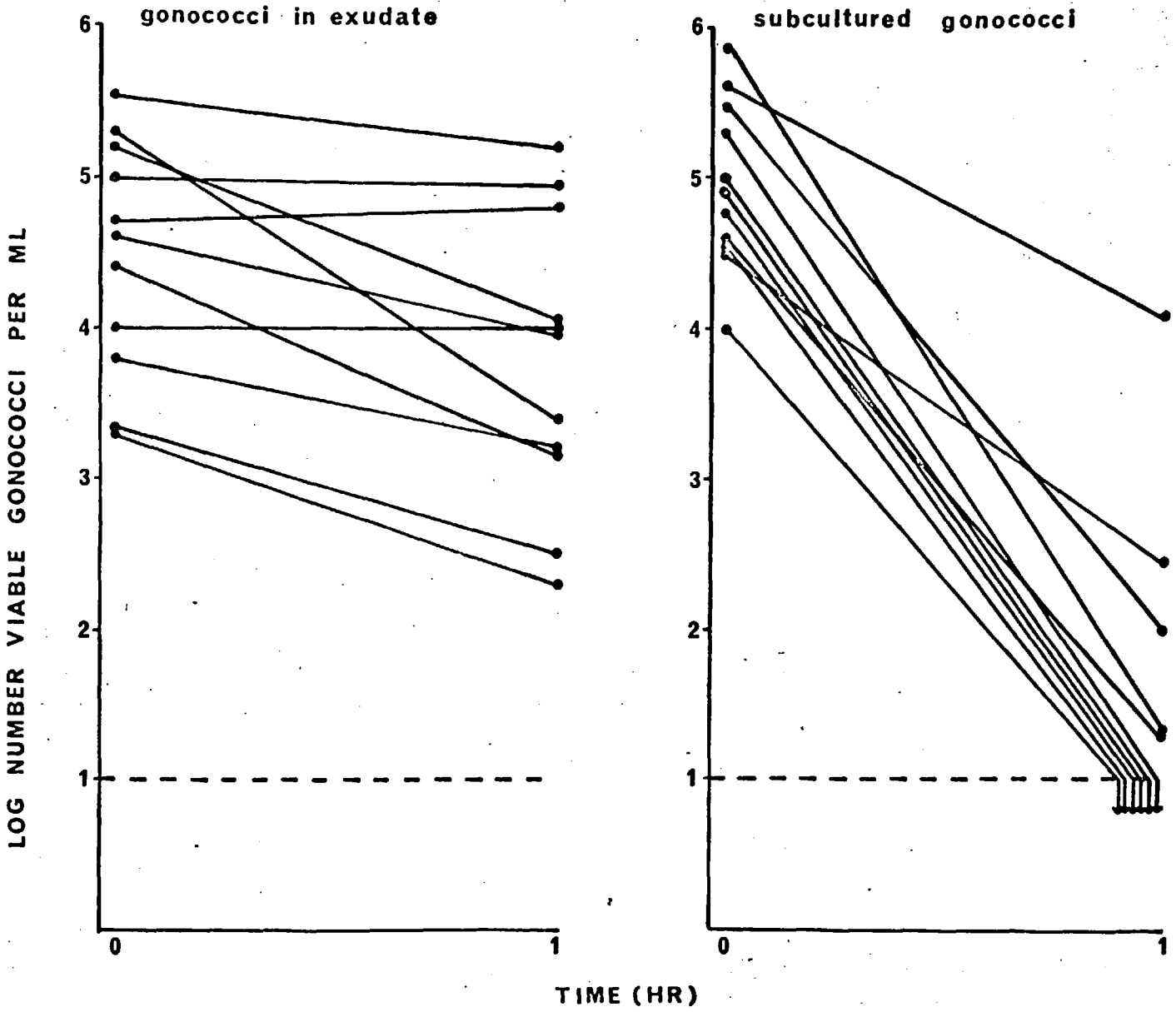
killing might be associated with the possession of a protective factor. If this is so it should also protect them from the bactericidal activity of complement and a pooled immune serum. To test this hypothesis the sensitivity of 10 of the strains of exudate gonococci used previously, and of the same strains after subculture to a normal human serum (MW) containing a 1:10 final concentration of pooled rabbit antigonococcal serum was examined as before (section 6.2.1.). The pooled serum was prepared by mixing equal volumes of the 10 rabbit antisera described in section 2.2.2. The results, summarised in Fig. 13, show that all but one (G87) of the subcultured gonococci was rapidly killed by complement plus pooled antigonococcal serum. However, the exudate gonococci remained more resistant. The log average fall in viable count of 0.7 (Table 27) was less than the log average fall in viable count of 1.5, presumably due to agglutination, when subcultured gonococci were exposed to the same concentration of antiserum in the absence of complement.

6.2.4. The effect of exudate on the serum killing of a streptomycin resistant gonococcal strain, G11.

The resistance of host grown gonococci could be explained if substances present in the exudate in some way interfered with serum killing. To test this possibility a subcultured gonococcal strain (G11) was selected which was resistant to more than 500 µg/ml of streptomycin incorporated in Amies and Garabedian medium. This strain was also insensitive to the bactericidal activity of normal human serum (MW), but was rapidly killed on the addition of immune sera (section 4.3.1.). After standardisation to the logarithmic growth phase in a biphasic flask containing 25µg/ml of streptomycin in the

Fig. 13.

The effect of polyvalent rabbit antiserum with human serum as a source of complement on strains of N.gonorrhoeae both in the native exudate and after subculture.


gonococci in exudate
subcultured gonococci
LOG NUMBER VIABLE GONOCOCCI PER ML
6
5
4
3
2
1
0
1
TIME (HR)

-113-

TABLE 27 The effect of complement and a pooled immune serum on the viability of exudate and subcultured gonococci.

Strain Number	Log_{10} Viable Gonococci						
	In Exudate			After subculture			
	Inoculum	Patients[a] serum	Patients[a] serum & antisera	Inoculum	Patients[a] serum	Patients[a] serum & antisera	Inactivated serum[a] & antisera
G70				4.8	3.2	2.5	
G71				4.9	2.7	1.7	
G85	5.0	4.8	5.0	5.5	2.0	2.0	3.5
G87	3.3	3.0	2.3	5.6	5.0	4.1	
G88	5.2	4.9	4.0	5.0	1.0	< 1.0	3.8
G89	4.4	4.6	3.2	4.5	3.4	2.5	
G90	5.5	5.3	5.2	4.0	4.3	< 1.0	
G100	4.0	4.5	4.0	5.3	< 1.0	< 1.0	3.8
G101	5.3	5.0	3.4	4.8	< 1.0	< 1.0	3.2
G102	4.7	4.8	4.0	4.9	4.2	< 1.0	
G103	3.9	3.6	3.2	5.2	1.0	1.3	
G104	3.3	3.2	2.5	4.5	2.8	1.3	3.4
MEAN:	4.5	4.4	3.7	5.0	2.5	1.3	3.5

overlying broth, 10^6 of the streptomycin resistant gonococci were incubated with 0.4 ml of normal human serum (MW) containing a final dilution of 1:10 of the pooled antigonococcal rabbit serum described in section 6.2.3. Viable counts were performed on the G11 inoculum at the start of the experiment, and on the number of gonococci surviving after incubation for 1 hr. The growth of exudate gonococci was suppressed by the use of media containing 25µg/ml of streptomycin. As the results summarised in Fig. 14 show, in 9 out of the 11 experiments G11 was rapidly killed, the average fall in viable count after 1 hr. incubation exceeding 5 log units. In the remaining two experiments using exudates G85 and G101 the gonococci recovered on the streptomycin plates at the end of the experiment were almost certainly derived from the exudates since they were of different colonial morphology to G11.

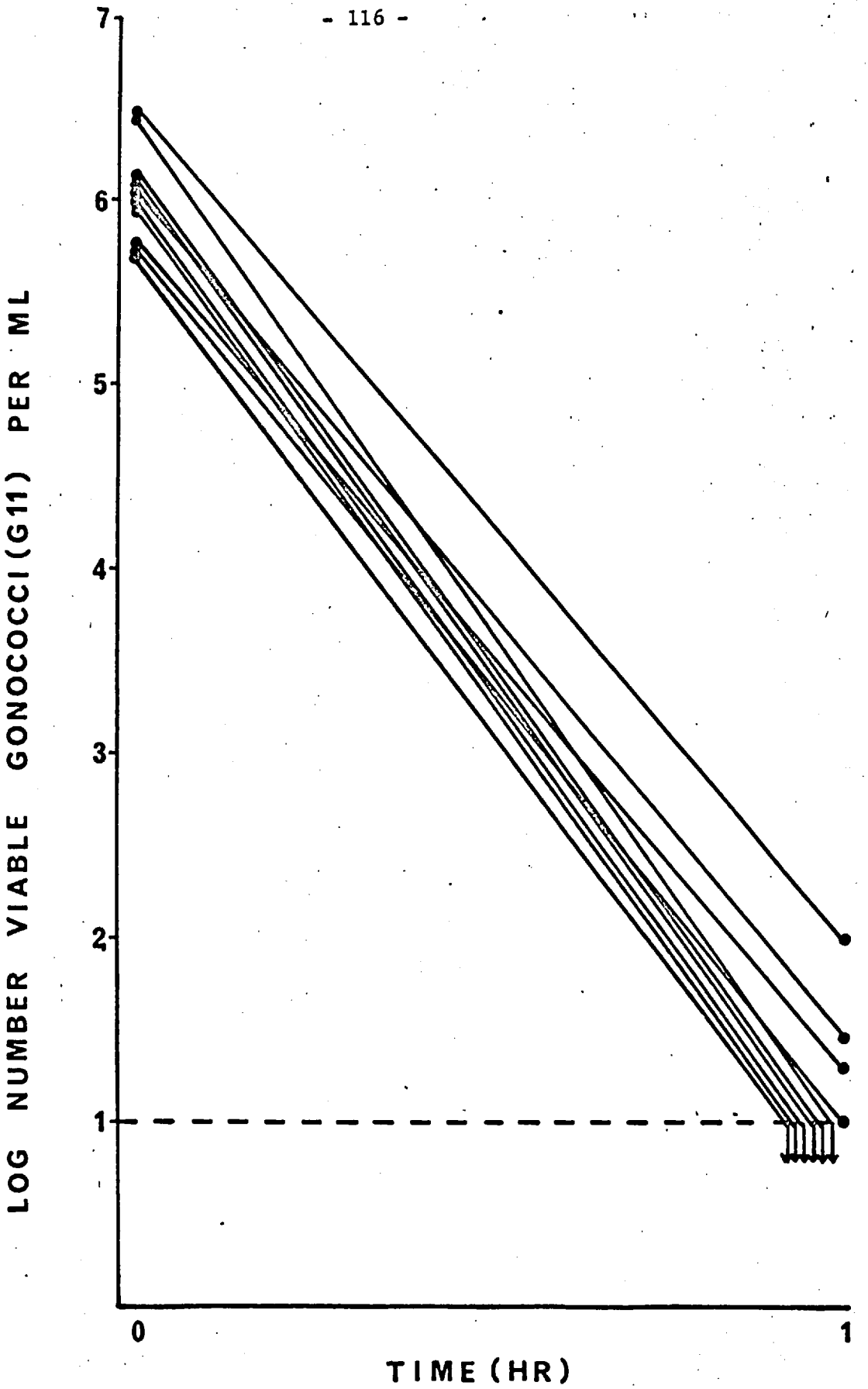
In a more rigorous series of experiments three heavy urethral exudates, each in a volume of 0.2 ml, were incubated for 6 hr. at 37°C with approximately 10^7 gonococci of strain G11 contained in 0.1 ml volumes. Pooled antigonococcal serum and complement was then added and the amount of serum killing assessed as previously. In each case the loss in viable count due to serum killing exceeded 3 log units. It is therefore unlikely that substances present in the exudate were anticomplementary or formed a protective coat around the gonococcus.

6.2.5. The effect of growth phase on the serum killing of subcultured gonococci.

Another possible explanation for the resistance of exudate gonococci could be that they were in an unsuitable growth phase for serum killing

Fig. 14.

The effect of rabbit antiserum plus complement on a streptomycin resistant strain of N.gonorrhoeae (G11 - colonial type 4) in the presence of urethral exudate. Viable counts were made on media containing 25µg/ml streptomycin.


LOG NUMBER VIABLE GONOCOCCI (G11) PER ML
7
6
5
4
3
2
1
0
1
TIME (HR)

when compared with the subcultured organisms, which were tested in the logarithmic phase of growth. To examine this possibility the same strains of subcultured gonococci from an 18 hr. culture on Amies and Garabedian medium were suspended in Dulbecco's solution. They were then tested as before for their sensitivity to a normal serum (MW) and the homologous patients' serum. It was expected that the gonococci growing on a plate would be in varying stages of growth. As the results in Table 28 show, the same strains from an 18 hour plate culture were more sensitive to the bactericidal action of a normal or the homologous patients' serum, than when they were tested in the logarithmic growth phase.

Analysis of urethral exudate by immunodiffusion.

6.3.1. The presence of serum bactericidal components in urethral exudate.

The resistance of gonococci to serum killing only becomes an essential attribute of virulence if it can be shown that they are exposed to serum factors *in vivo*. To confirm the presence of serum antibodies and complement urethral exudates were examined by immunodiffusion and immunoelectrophoretic techniques.

Urethral pus from patients with acute gonorrhoea were suspended in 0.5 ml of Dulbecco's solution. Insoluble components were removed by centrifugation and the supernatant tested by immunodiffusion on microscope slides using 1% w/v agar in saline as the supporting gel. Fig. 15a shows the results of an Ouchterlony test with 8µl of urethral exudate supernatant in the centre well, and 8µl volumes of various commercial rabbit antisera to human serum components in the outer well. The resulting precipitin lines which developed after overnight incubation at room temperature show

TABLE 28

Comparison of the effect of growth phase on the killing of subcultured gonococci by a normal serum (MW) and homologous patients' sera.

Strain Number	Log_{10} number of viable gonococci in:					
	Logarithmic growth phase(broth)			Mixed growth phase(plate)		
	Inoculum	Patients[a] serum	Normal serum	Inoculum	Patients[a] serum	Normal[a] serum
G70	4.8	3.2	3.3	6.5	1.3	1.0
G71	4.9	2.7	2.2	5.8	<1.0	<1.0
G72	4.2	2.3	2.2	6.1	2.8	1.6
G73	5.0	<1.0	<1.0	5.2	<1.0	<1.0
G74	3.4	<1.0	1.0	4.3	2.1	2.6
G77	2.8	1.0	2.7	4.0	<1.0	<1.0
G78	2.6	1.0	2.5	4.3	2.3	2.3
G79	4.6	2.5	2.3	6.4	2.3	2.3
G83	5.4	<1.0	<1.0	4.4	1.0	1.0
G89	4.5	3.4	3.7	4.6	<1.0	3.0
G102	4.9	4.2	2.5	5.5	2.8	1.0
G104	4.5	2.8	3.3	5.7	1.9	2.5
MEAN	4.3	1.9	2.1	5.2	1.4	1.6
MEAN KILLED		2.4	2.2		3.8	3.6

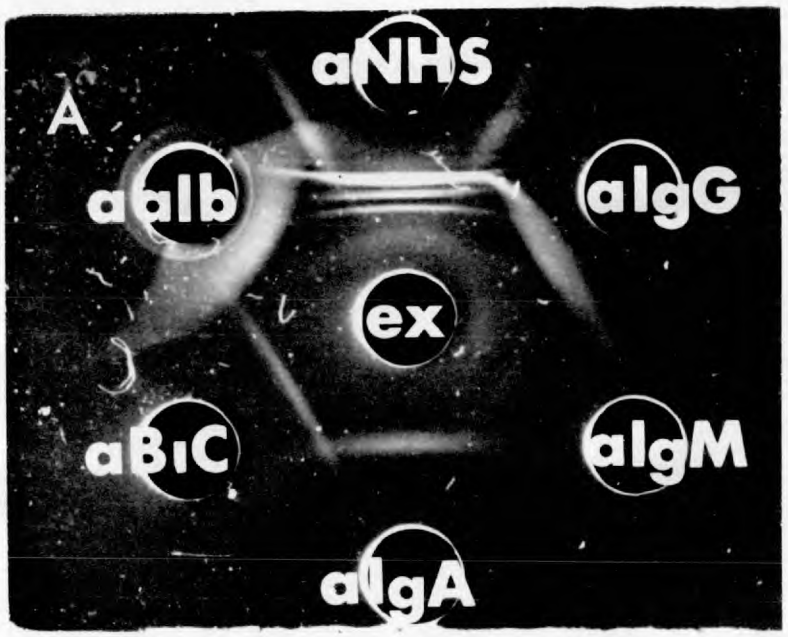
a = Incubated for 60 min. at 37°C.

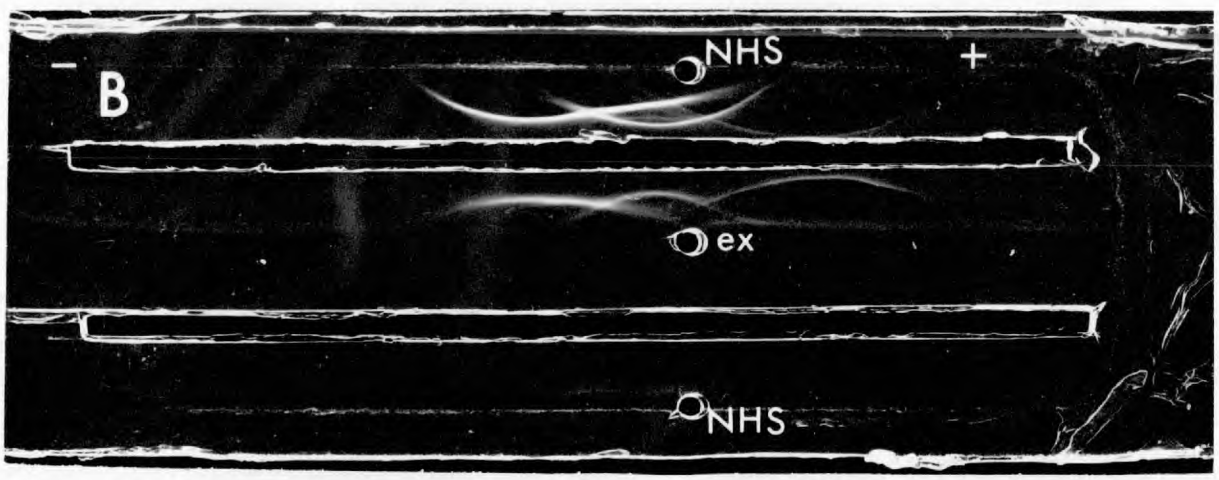
Fig. 15.

Qualitative analyses of urethral exudate by microimmunodiffusion and microimmunoelectrophoresis.

A. Immunodiffusion. Gonococcal exudate in the central well and various antisera to human serum components in the outer well. The results confirm the presence of immunoglobulins and complement in gonococcal exudate.

B. Immunoelectrophoresis. The upper trough contains Coombs reagent (anti human immunoglobulins) whilst the lower trough contains anti human IgM. The electrophoretic mobility of the immunoglobulins in gonococcal exudate are the same as the immunoglobulins in the normal human serum.


A
aNHS
aalb
algG
ex
aBIC
algM
algA


B
NHS
+
ex
NHS

the presence in the exudate of IgG, IgM, IgA and beta-1C/beta-1A. The presence of albumin in the urethral pus strongly suggests, as expected, that it is derived from serum as part of an inflammatory exudate. Similar results were obtained with supernatants from 20 other urethral exudates. The faintness of the precipitin line corresponding to the presence of IgM is almost certainly due to the low potency of the antiserum used since the same exudate gave a much better precipitin line with a different anti IgM serum.

Electrophoresis of 2µl volumes of urethral exudate (in the centre well) and normal human serum (in the outer well) was carried out on a microscope slide using 1% w/v Oxoid Ionagar No. 1 as the supporting medium. The samples were run in 0.025 M veronal acetate buffer pH 8.2 for 4 hr. at a potential gradient of 1 V./cm. Specific rabbit anti human IgG or anti human sera were added and the precipitin lines developing after overnight incubation at room temperature noted (Fig. 15b). As expected, the electrophoretic pattern and mobility of the components present in urethral exudate were similar to those of normal human serum.

The detection of complement with an anti beta-1C/beta-1A serum does not necessarily mean that it is present in an active form. In an attempt to resolve this point, four urethral exudates from patients with acute gonorrhoea were collected into 0.4 ml volumes of ice cold 'Oxoid' complement diluent. After centrifugation 0.1 ml volumes of the exudate supernatants were incubated at 37°C for 1 hr., and then overnight at 4°C in a haemagglutination tray with 0.1 ml volumes of a 0.5% v/v suspension of sheep red cells which had been optimally sensitised with rabbit anti sheep cell haemolysin (Staynes). No haemolysis was detected in any of the wells.

This is perhaps not surprising, since the exudate itself may well have been in the urethra for a considerable period of time before being sampled. Nevertheless, despite this, it seems highly likely that at the site of gonococcal infection where the inflammatory reaction occurs (Harkness, 1948) the organisms are exposed to the bactericidal components of serum.

CHAPTER 7. The maintenance and assessment of gonococcal virulence in vitro.

7.1.1. Introduction.

The number of experiments which are possible using host grown gonococci as a source of virulent organisms is very limited. Such experiments are at the mercy of the irregular supply of patients, whilst the organisms themselves are difficult to obtain free of contaminating host derived materials and are not available in sufficient numbers for immunochemical characterisation. Ultimately it is vital that a way be found to maintain virulent gonococci in the laboratory.

It is probable that the avirulence of primary gonococcal isolates in laboratory culture is a phenotypically induced alteration. Cultivation of gonococci in animals or the chick embryo might therefore provide the necessary environment to reverse this change. The report of Walsh et al. (1963) that the 8 day embryonated hen's egg is capable of distinguishing between gonococci which were virulent or avirulent for human volunteers is therefore of interest in this connection.

This chapter describes some preliminary experiments, some of them performed before the resistance of virulent gonococci to serum killing had been discovered, designed to examine these possibilities.

7.2.1. Susceptibility of gonococci of different colonial types to serum killing.

Urethral exudates were collected from men with acute gonorrhoea. After homogenisation with a Teflon grinder and washing (section 6.1.2.)

they were suspended in 0.5 ml of Dulbecco's solution and tested for their susceptibility to killing by 4 different normal sera as described in section 6.2.1. Viable counts were performed on Kellogg's colonial type indicator medium. This consists of Difco GC medium base to which is added: dextrose 4 mg/ml, glutamine 100µg/ml, ferric nitrate 10µg/ml, and cocarboxylase 0.2µg/ml (White and Kellogg, 1965). After overnight incubation for 18 hr. five microcolonies of the same colonial type were selected under a dissecting microscope, picked off with a loop, and tested as before for their susceptibility to serum killing. All of the primary isolates consisted of colonial types 1 or 2. Each isolate was then selectively cultured as described by Kellogg et al. (1963) so as to obtain virtually pure cultures of colonial types 1 and 2 or 3 and 4. After each culture they were retested for their sensitivity to serum killing, whilst portions were preserved with no detectable loss in viability by freezing in liquid nitrogen (sections 2.1.4., 3.2.2.). This permitted repetition of the experiment if required. N.gonorrhoeae strain F62, which was kindly provided by Dr. Alice Reyn, originated from Kellogg's laboratory and is of particular interest since it is the strain for which he described an association between colonial type and virulence. Immediately on receipt the lyophilised ampoules of F62 were cultured on Kellogg's medium, checked after 18 hr. incubation for their colonial types, and the cultures preserved by freezing in liquid nitrogen. The serum sensitivities of F62 colonial types 1 and 2, or 3 and 4, were then tested as previously described (section 6.1.2.).

Table 29 shows the sensitivity of 5 strains of gonococci in urethral

TABLE 29

Killing of gonococci of different colonial types by normal and immune sera. This table compares the sensitivity of 5 strains of gonococci present in the urethral exudate and after serial subculture to killing by 4 different normal sera and a pooled gonococcal antiserum.

EXUDATE 1

No. of subcultures	Colonial type	Inoculum count	Log_{10} Viable Count after incubation for 1 hr. in:					
			Normal Human Sera				Pooled antisera	DSB-s broth
			PW	MW	TM	BD		
Exudate		4.8	4.7	4.5	4.5	4.6	ND	4.6
1	1 & 2	7.3	5.6	4.4	4.7	4.5	4.1	6.8
2	1 & 2	6.5	3.5	<3.5	<3.5	<3.5	<3.5	6.1
3	1 & 2	7.2	<3.5	<3.5	3.5	3.5	<3.5	7.1
3	3 & 4	6.6	3.5	3.5	4.0	3.5	<3.5	7.2

EXUDATE 2

No. of subcultures	Colonial type	Inoculum count	Log_{10} Viable Count after incubation for 1 hr. in:					
			Normal Human Sera				Pooled antisera	DSB-s broth
			PW	MW	TM	BD		
Exudate		4.5	4.4	4.4	4.0	4.4	ND	4.2
1	1 & 2	6.9	6.9	6.0	6.5	4.6	3.5	6.1
2	1 & 2	5.9	4.2	3.5	3.5	3.5	3.5	5.5
3	1 & 2	7.1	6.6	5.8	6.6	6.0	<3.5	7.0
3	3 & 4	7.3	6.6	6.4	6.6	6.0	<3.5	7.3

TABLE 29 (cont'd)

EXUDATE 3

No. of subcultures	Colonial type	Inoculum count	Log_{10} Viable Count after incubation for 1 hr. in: Normal Human Sera PW	MW	TM	BD	Pooled antisera	DSB-s broth
Exudate		5.9	5.2	5.3	5.2	5.2	5.0	5.3
1	1 & 2	6.1	3.5	3.5	< 3.5	< 3.5	< 3.5	6.5
2	1 & 2	6.7	4.0	4.0	3.8	3.5	< 3.5	6.4
2	3 & 4	5.5	4.3	4.0	3.5	4.3	4.3	5.1

EXUDATE 4

No. of subcultures	Colonial type	Inoculum count	Log_{10} Viable Count after incubation for 1 hr. in: Normal Human Sera PW	MW	TM	BD	Pooled antisera	DSB-s broth
Exudate		5.9	5.3	5.4	5.3	5.2	5.0	5.4
1	1 & 2	7.3	6.4	5.9	6.3	6.2	4.6	7.2
2	1 & 2	6.0	3.5	3.6	4.1	3.6	<3.5	6.1

EXUDATE 5

No. of subcultures	Colonial type	Inoculum count	Log_{10} Viable Count after incubation for 1 hr. in: Normal Human Sera PW	MW	TM	BD	Pooled antisera	DSB-s broth
Exudate		4.4	4.1	4.1	4.3	4.3	ND	4.3
1	1 & 2	6.0	5.6	4.3	4.5	4.3	4.0	5.8
2	1 & 2	4.8	4.0	3.9	4.0	4.2	3.7	4.8
3	1 & 2	6.9	6.3	5.7	6.1	6.2	<3.5	6.5
3	3 & 4	6.6	6.8	6.0	6.6	6.9	3.7	7.0

TABLE 29 (cont'd)

F62

No. of subcultures	Colonial type	Inoculum count	Log10 Viable Count after incubation for 1 hr. in:					
			Normal Human Serum				Pooled antisera	DSB-s broth
			PW	MW	TM	BD		
Large	1 & 2	7.1	3.8	3.5	3.5	3.5	< 3.5	6.7
Large	3 & 4	6.7	< 3.5	< 3.5	< 3.5	< 3.5	< 3.5	6.5

exudate to killing by various normal and immune sera, together with the sensitivity of the cultured gonococci of different colonial type derived from them. As before all 5 strains of gonococci present in the host's urethral exudate were completely resistant to killing by any of the 4 normal human sera tested. However, after 18 hr. cultivation on Kellogg's medium they became noticeably more susceptible to serum killing even though they were of colonial types 1 and 2. After further subculture it became possible to select pure colonial types 3 and 4 from the cultures. However, there were no noticeable differences in susceptibility to serum killing between colonial types 1 and 2 or 3 and 4. All the colonial types of strain F62 were highly susceptible to killing by the 4 normal human sera tested. If gonococci of colonial types 1 and 2 were fully virulent it would have been expected that, like the host grown organisms, they would be more resistant to serum killing. It therefore seems unlikely that the virulence of gonococci can be maintained by colony typing.

7.3.1. The multiplication of gonococci in the chick embryo.

To test whether the 8 day embryonated hen's egg was capable of selecting for virulent gonococci its ability to support the growth of three different strains of gonococci was compared. Strain G9 was a fresh primary gonococcal isolate and presumably consisted mainly of colonial type 1 organisms. G2 was an old laboratory strain consisting of colonial types 3 or 4 and presumably avirulent. Strain G1 was a fresh gonococcal isolate which had been passaged eleven times without

subculturing through the chorioallantoic cavity of 8 day chick embryos.

Just prior to the experiment antibiotic-free 8 day old chick embryos were candled to ensure their viability. An area of the chorioallantoic membrane which was relatively free of blood vessels was then marked. Each strain was grown in a biphasic flask to the logarithmic phase and a viable count of approximately 10^9/ml. Doses corresponding to between 10^1 and 10^7 organisms in 0.1 ml volumes of DSB-s were injected at the point marked into the chorioallantoic cavity using 5 eggs for each dose. They were then incubated at 36°C and candled for viability 24, 48, 72 and 144 hr. after the experiment. Samples of chorioallantoic fluid were also removed aseptically from one egg out of each group of five at 24 and 144 hr. and the number of gonococci determined by viable counting. The results are shown in Table 30.

Inoculation of any of the three strains in doses as low as approximately 10 organisms led to the ready multiplication of gonococci in the chorioallantoic fluid, the number of viable organisms being approximately 10^8/ml after 24 hr. incubation. However, despite the presence of such a large number of gonococci in the chorioallantoic fluid, a considerable number of embryos remained viable at least 6 days after the experiment whether gonococci were still present or not. A large number of leukocytes were present in the chorioallantoic fluid of infected eggs, but it was not possible microscopically to see any phagocytosis.

These findings are contrary to those of Walsh et al. (1963) who reported that it was only possible to detect the growth of virulent gonococci in the chick embryo after the infected chorioallantoic fluid

TABLE 30

Pathogenicity of gonococci via the chorioallantoic route for the 8 day chick embryo.

Dose of viable gonococci	Number of embryos dead (out of 5) after 144 hr.			Log_{10} number of viable gonococci/ml[c] chorioallantoic fluid after 24 hr.		
	Strain: G1	G2	G9	Strain: G1	G2	G9
10^1	4	0	0	8.0[b]	6.3	5.0
10^2	1	1[a]	1	8.8	8.0	7.8
10^3	1	2	1	7.5	8.6	4.8
10^4	2	4	1	ND	ND	ND
10^5	2	2	0	ND	ND	ND
10^7	0[a]	5	3	ND	ND	ND

a Three eggs only in these groups.

b This embryo was dead at 24 hr.

c One embryo from each group of five randomly selected for counting.

had been passaged a number of times through the chorioallantoic cavity of uninfected eggs. The ability of an old laboratory gonococcal strain to proliferate rapidly in the embryonated egg discouraged attempts to use this technique as a method for maintaining virulent gonococci.

Attempts to maintain gonococci in experimental animals.

7.4.1. Choice of laboratory animal.

The choice of the mouse as the experimental animal of choice was governed by the following factors. Firstly, its mean normal temperature is within 1°C of that of man and is lower than that of most other laboratory animals. The optimum growth temperature of gonococci is considered to be slightly under normal human temperature (Hill et al., 1945). Secondly, mice are of sufficiently low cost to permit their use in large numbers where necessary. Finally, it is clear from the work of Miller (1944) using the intraperitoneal mucin technique that given the right conditions mice are susceptible to gonococcal infection.

7.4.2. Growth of gonococci in the mouse peritoneum.

If mice are potentially susceptible to gonococcal infection elimination of their defence mechanisms should enable the gonococci to multiply. To test this hypothesis gonococci were suspended in Dulbecco's solution containing 0.1% w/v purified bovine serum albumin. The suspensions were then aseptically sealed inside sacs of 'Visking' dialysis tubing or diffusion chambers of membrane filters (Millipore, 0.45μ). The sacs or chambers were then introduced into the peritoneum

of anaesthetised large adult mice by means of an incision in the abdominal wall which was later sutured. Twenty four hours later the mice were killed, the abdominal cavity opened, and the contents of the sacs cultured. The results of these experiments are shown in Tables 31 and 32. The strains used were freshly isolated gonococci which had been preserved by lyophilisation. In all cases the initial inoculum 24 hr. later had multiplied, despite the presence in the Millipore chambers of macromolecules. The species specificity of subcultured gonococci is therefore not solely explained by environmental or nutritional factors.

7.4.3. The possible use of immunosuppressant drugs for modifying the resistance of mice to experimental gonococcal infection.

The ability of gonococci to grow in the mouse peritoneum within a Millipore chamber supports the belief that mice are potentially susceptible to gonococcal infection, provided that the appropriate defence systems can be sufficiently inactivated. The possible use of immunosuppressant drugs to achieve this was therefore investigated. Doses of 2-8 mg. of cortisol acetate were injected at 12 hourly intervals intraperitoneally into 15 day old female mice for 2 days prior to the experiment. 15 day old female mice were chosen because the vaginal epithelia have not yet become overlaid with squamous cells (Powierza 1912, cited by Hill et al., 1965). A small rounded sterile glass tube was smeared with gonococci from an overnight culture on Amies and Garabedian medium, inserted into the vagina of an anaesthetised mouse, rotated, and withdrawn. Twenty four hours later the mouse was killed and samples for staining and culture taken

TABLE 31

Survival of gonococci in the mouse peritoneum within 'Visking' sacs.

Mouse Number	Strain	Log_{10} Viable Count of Gonococci in: Inoculum	Visking Sac at 24 hr
1	G9	6.0	8.0
2	"	"	8.0
3	"	"	7.6
4	"	"	8.0
MEAN	G9	6.0	7.9
1	G11	5.0	8.5
2	"	"	8.7
3	"	"	8.6
MEAN	G11	5.0	8.6
4	G11	6.0	8.9
5	"	"	8.5
6	"	"	8.3
MEAN	G11	6.0	8.6

TABLE 32

Survival of gonococci in the mouse peritoneum within 'Millipore' chambers, 0.45μ.

Mouse Number	Strain	Log_{10} Viable Count of gonococci in Inoculum	Millipore chamber at 24 hr.
1	G9	6.0	8.0
2	"	"	5.9
3	"	"	7.5
4	"	"	8.0
MEAN	G9	6.0	7.3
1	G11	5.5	7.5
2	"	"	7.9
3	"	"	6.5
4	"	"	6.8
5	"	"	6.6
MEAN	G11	5.5	7.0
1	G71	5.7	8.0
2	"	"	8.0
3	"	"	7.7
4	"	"	7.3
5	"	"	8.7
MEAN	G71	5.7	7.9

from the vagina with a similar glass tube. Control mice which had been inoculated with gonococci but were not given cortisol acetate showed the presence of polymorphonuclear cells with extra- and intra-cellular gonococci in the vaginal smears. These organisms, however, must have been dead since they were not recoverable by culture. Mice which had been infected with gonococci and treated with cortisol acetate also showed polymorphonuclear cells with intra- and extra-cellular gonococci in the smears. However, in most cases the cellular response was much less, presumably due to the anti-inflammatory activity of cortisol, but again no gonococci were recoverable 24 hr. after inoculation. Vaginal smears from steroid treated mice only, frequently showed heavy growths of mixed organisms.

The effect of cortisol acetate on the survival of gonococci in neonatal mice was also investigated. Two day old mice weighing between 1 and 2 grams were given a single injection subcutaneously in the dorsal neck region of 0.2 mg. cortisol acetate/gram body weight contained in 0.05 ml saline. These mice subsequently developed a fatal wasting disease with diarrhoea, failure to gain weight, and loss of hair (see Fig. 16). The majority of deaths occurred before they were 12 days old and have been associated by Jutila and Reed (1968) with severe immunosuppression leading to a fatal infection by their own normally benign gut organisms. Nevertheless subcutaneous or intraperitoneal inoculation of up to 10^7 freshly isolated gonococci or of gonorrhoeal exudate between 24-144 hr. after the cortisol acetate injection did not induce a more rapidly fatal infection nor were gonococci recoverable by culture 24 hr.

Fig. 16.

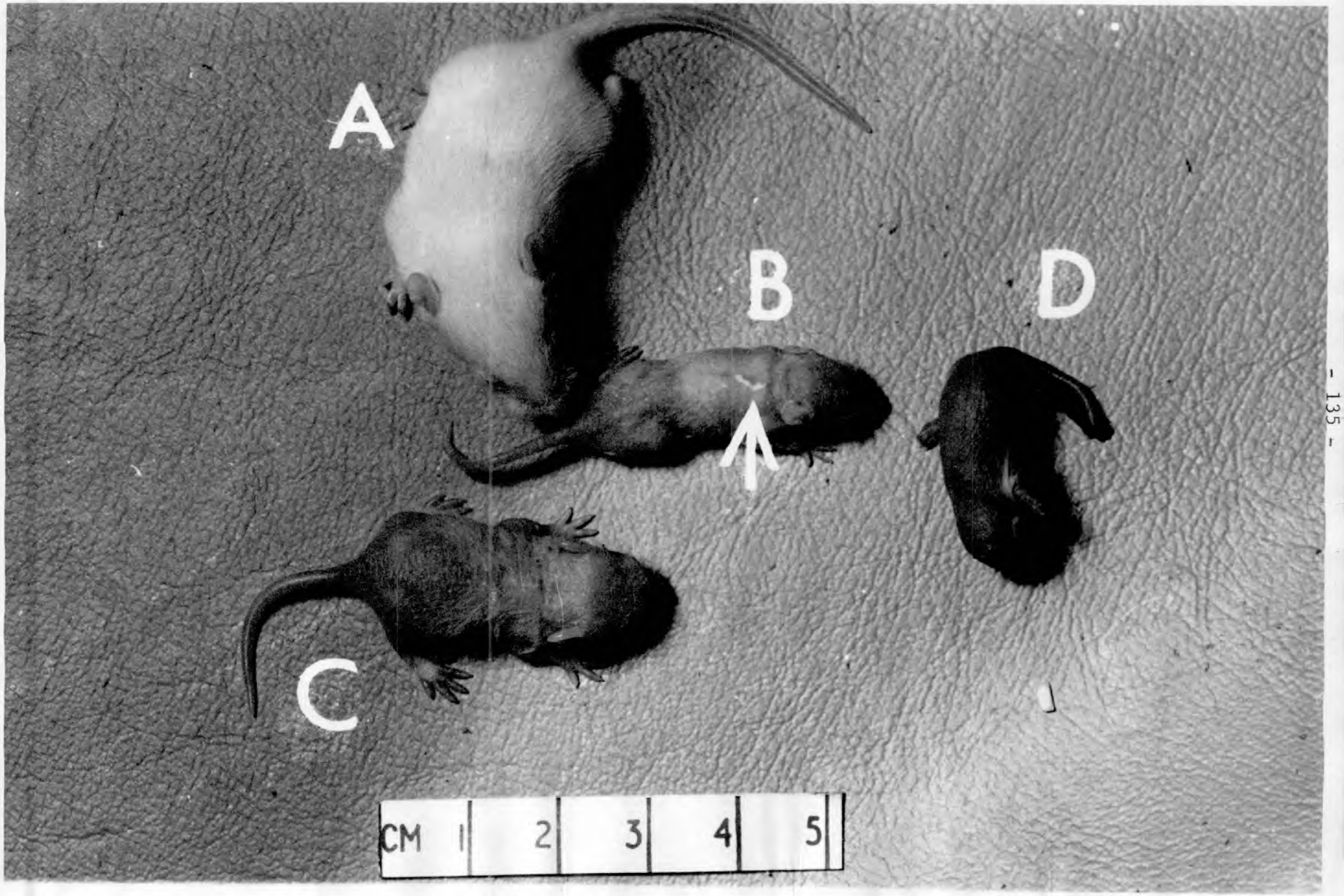
Cortisol acetate induced wasting disease in neonatal mice.

A. Control 11 day old mouse injected when 2 days old with 0.05 ml saline.

B. 11 day old mouse injected when 2 days old with 0.2 mg cortisol acetate/gram body weight in 0.05 ml saline. The cortisol acetate is still visible under the dorsal neck skin (arrow). This mouse died 5 hr. after the photograph was taken.

C. 11 day old mouse injected when 2 days old with 0.2 mg cortisol acetate/gram body weight. This mouse survived.

D. 2 day old uninjected mouse.


A
B
D
C
CM 1 2 3 4 5

later. Methatrexate, a folate antagonist which in rats can produce total depletion of polymorphonuclear cells (Willoughby and Giroud, 1969) was also unsuccessful in that the doses required to deplete circulating polymorphs significantly in neonatal or adult mice proved to be toxic.

Attempts to increase the virulence of gonococci for mice by passage within the peritoneum suspended in hog gastric mucin (Miller, 1944) failed because of the toxicity for gonococci of the batches of mucin available. Similarly, when approximately 10^6 freshly isolated gonococci (strain G71) were injected intraperitoneally into mice suspended in 1 ml of 5% fresh haemoglobin solution, gonococci did not persist at 24 hr. even though, with other bacteria, iron containing compounds have been shown to enhance their virulence (Bullen et al., 1968). However, in both these experiments large numbers of viable gonococci derived from the peritoneum rapidly appeared in the blood.

Clearly there are a large number of other possible techniques for modifying the host's resistance to experimental infection. However, the artificiality of such infections, the lack of success of these experiments, and the finding that gonococci grown within the mouse peritoneum in Visking sacs were serum sensitive, discouraged this approach to the problem of maintaining virulent gonococci in the laboratory.

CHAPTER 8. Discussion.

Antigenic structure.

Using a bactericidal assay it was possible to divide 60 strains of gonococci into 4 main groups according to their sensitivity to killing by normal human or immune rabbit antibodies and complement. These groups were not entirely homogeneous and no doubt additional subdivisions could be detected with further work along the same lines. The antigens involved in serum killing are therefore heterogeneous.

About a third of the 60 strains tested were sensitive to killing by a normal human serum (MW). The bactericidal antibodies in this serum were probably formed against cross reacting antigens on organisms present in the normal flora, since they could be absorbed out with other Neisseria or even by members of other genera. This situation is unlike that in meningitis where specific bactericidal antibodies arise as a result of nasopharyngeal colonisation (Goldschneider et al., 1969b). The fact that the antibodies concerned were produced against a wide variety of antigens could explain the varying ability of normal sera to kill gonococci of different antigenic specificities.

With other Gram negative bacteria antibody to the lipopolysaccharide is bactericidal (Felix and Olitzki, 1926). Since the gonococcal antigens involved in serum killing were resistant to trypsin and to autoclaving at 121°C it seemed possible that they might also be lipopolysaccharides. This was subsequently tested by absorption or neutralisation experiments with purified gonococcal lipopolysaccharide. When anti G1 or anti G50 sera were absorbed with sheep red cells coated with G1 or G36

lipopolysaccharides no specific antibody to these antigens could be detected in a haemagglutination test. However, these same absorbed sera had markedly reduced or absent bactericidal activity against a number of cross reacting gonococcal strains. The haemagglutinating and bactericidal activity of control sera absorbed with unsensitised red cells was not significantly affected. Again the bactericidal activity of anti G1 serum for strains G11 and G29 could be totally abolished by neutralisation with as little as 1-4μg of purified G1 lipopolysaccharide. Nevertheless it seems probable that antigens other than lipopolysaccharide are also involved in the serum killing of gonococci. Thus absorption or neutralisation of anti G1 serum with G1 lipopolysaccharide abolished the serum's bactericidal activity for strain G29 without completely removing its ability to kill the homologous strain. By contrast, the bactericidal activity of anti G1 serum for strains G1 or G29 could be removed by absorption with the homologous heat treated ($100^{o}C$) organism.

Since lipopolysaccharide is involved in serum killing it must carry the type or group specific immunodeterminants responsible for the heterogeneity of gonococcal antigens in the serum bactericidal reaction. The widely differing ability of an antigonococcal serum to agglutinate red cells coated with lipopolysaccharides from different strains shows that these antigens do have type or group specificies. This has been independently confirmed by Maeland (1969). Comparison of the results of the haemagglutination and bactericidal tests showed that both normal and immune sera reacted with a wider range of organisms in the haemagglutination than in the bactericidal test. It might be that the mild alkali

treatment of the lipopolysaccharide required to fix them onto red cells in some way reduced the antigens' serological specificity. However, although alkali treatment of lipopolysaccharide is known to remove O acetyl groups, alter its molecular weight and abolish its toxicity (Davies et al., 1958; Tripodi and Nowotny, 1966) there is no evidence that its serological specificity is altered (Neter et al., 1956). It seems more likely therefore that in the intact organism the access of antibody or complement to the lipopolysaccharide is restricted, possibly due to the presence of blocking antigens.

The epidemiological study of gonorrhoea is handicapped by the absence of a suitable method for typing N.gonorrhoeae. Attempts to serotype gonococci have been frustrated because the organisms are often autoagglutinable (Lind, 1967) whilst antisera which have been absorbed for use in the complement fixation test are frequently anticomplementary (Reyn, 1949b and c). The fact that gonococci could be divided into a number of serotypes using a bactericidal test suggests a possible means of classifying N.gonorrhoeae for epidemiological research. Although bactericidal tests are technically difficult Gold and Wyle (1970)have independently advocated this system for epidemiological research on meningococcal infections. As lipopolysaccharides are involved in the bactericidal reaction and possess type specificity, it should be possible to develop a serological classification based on this antigen. However, the outcome of the bactericidal test is probably the result of the interaction of lipopolysaccharide and blocking antigens. It is therefore unlikely that the bactericidal test would give identical results to any

other method based on gonococcal lipopolysaccharide. Nevertheless, similar results to the bactericidal test might be obtained more practicably by incubating gonococcal strains on slides with the various antisera and a complement source. Those strains which have reacted with the antisera and bound complement should then be detectable after specific staining with fluorochrome-conjugated anti complement sera. Thus Goldschneider et al. (1969a) found that the results of bactericidal tests with human serum against meningococci were closely paralleled by the results obtained with an indirect fluorescent antibody test.

Ideally antigens used for taxonomy or diagnosis ought ultimately to be chemically defined. The finding that in the haemagglutination test gonococcal lipopolysaccharides only cross reacted with antisera to meningococci or gonococci suggests that it has a relatively unique structure. However, the structure of the type specific immunodeterminants on the antigen remains unknown. The agglutination of G36 lipopolysaccharide coated red cells by anti G37 serum could be inhibited by galactose or, more strongly, α or β galactosyl (1-4) glucose (α or β lactose). It was not inhibited by α galactosyl (1-6) glucose (α melibiose). These results are similar to those described by Maeland (1969b). Therefore one can postulate that the immunodeterminant involved contains a galactose residue whose specificity is determined by a 1-4 linkage but which is independent of its anomeric configuration. Nevertheless, final concentrations of at least 0.07 M α or β lactose were required to demonstrate significant inhibitory activity. This seems a surprisingly high concentration if a 1-4 linked galactose is part of the immunodeterminant when compared with,

for example, the 2 micromoles of N-acetyl glucosamine required to inhibit the precipitin reaction between group A streptococcal carbohydrate and homologous rabbit antiserum (McCarty, 1958). However, it is unlikely that the result was due to experimental artifact since final sugar concentrations as high as 0.28 M did not inhibit the agglutination of red cells coated with G1 or G2 lipopolysaccharides.

A simple method for diagnosing gonorrhoea which does not require genital examination is urgently required if the disease is to be controlled by the detection of asymptomatic reservoirs of infection in the population (WHO, 1965). Until recently, it was not clear whether specific antibodies are produced early in acute gonorrhoea. However, using a haemagglutination test antibodies to gonococcal lipopolysaccharide were found in up to 80% of the sera of patients with gonorrhoea and less than a quarter of normal control sera. This finding is in agreement with the results of Wallace et al. (1970) who found antibodies to gonococcal lipopolysaccharide in a bentonite flocculation test in approximately 78% of patients with gonorrhoea. However, the full details of this work are not yet available and the degree of cross reactivity is unknown. In a series of publications a group of Indian workers (Nair and Chacko 1966a and b; Chacko and Nair, 1969) described a microprecipitin test which detected antibodies in 95-100% of patients with acute gonorrhoea but did not cross react with normal control sera. Nevertheless, as the authors themselves admit, other investigators have found it difficult to reproduce the same results (Nair and Chacko, 1966b). It is not clear that the antigen reactive in the microprecipitin test is lipopolysaccharide as these workers suggest

because it did not produce a Shwartzman reaction (Nair and Chacko, 1966a).

The chemical nature of the protoplasmic antigen active in the promising serotest described by Reising et al. (1969) is also unknown. Moreover, it seems that the antigen involved has some type specificity because certain patients' sera gave a positive result only with antigen isolated from their own gonococcal strain (Danielsson et al., 1970). Obviously then, there is no completely satisfactory serotest for the diagnosis of gonorrhoea. However, it is hoped to develop tests using gonococcal lipopolysaccharide which would take advantage of the frequent occurrence of antibodies to this antigen in early gonorrhoea yet eliminate the cross reactions with normal sera.

Virulence.

Antibodies present in normal human sera are bactericidal for subcultured gonococci (Spink and Keefer, 1937) but man is uniquely susceptible to gonorrhoea. One possibility is that gonococci growing in the host are not exposed to the serum bactericidal components. This is unlikely since gonococci can persist in urethral pus despite the fact that immunoglobulins and complement were demonstrable by immunodiffusion. The natural antibodies in normal human serum which were bactericidal for subcultured gonococci were found to be strain specific. It might therefore be that gonococci only infect individuals lacking antibody to the strain concerned. This is also unlikely in view of the enormous incidence of gonorrhoea and the high rate of infections amongst contacts of patients with culturally proven gonorrhoea (Holmes et al., 1970). Alternatively it may be that gonococci growing in the host are protected from serum

killing. Evidence was obtained to suggest that this is so.

When gonococci present in exudate and the same strain after minimal subculture were tested for their sensitivity to killing by the homologous patients' sera, it was found that gonococci grown in the host were totally resistant to serum killing, whereas in 85% of cases the same strain after subculture was rapidly killed. The possibility that substances present in the exudate were interfering with serum killing was excluded because the serum sensitivity of a streptomycin resistant strain of gonococcus was unimpaired after incubation with heavy urethral exudates for up to 6 hr. Similarly, the possibility that the subcultured gonococci were sensitive to serum killing because they were tested in a particularly susceptible growth phase is also unlikely, because gonococci from an 18 hr. plate culture which were presumably in a mixture of growth phases were also highly sensitive.

The resistance of gonococci which had been grown in the host to the bactericidal activity of natural or immune antibodies and complement can be explained in two ways. It could be that gonococci in the host are resistant because they possess a greater amount of an antigen which is, nevertheless, also present in lesser quantity on subcultured gonococci. Thus for Escherichia coli it is known that decreasing amounts of lipopolysaccharide can be correlated with increasing serum sensitivity (Finegold, 1970) so it might be that subcultured gonococci contain decreased amounts of lipopolysaccharide comparable to the smooth to rough transformation in the Enterobacteriaceae.

The second possibility is that gonococci growing in the host possess

a protective factor, not present in subcultured organisms, which blocks the access of antibody to the underlying sensitive lipopolysaccharide. This factor could be a surface antigen comparable to the K antigens of E.coli or the Vi antigens of Salmonella typhi which enable organisms possessing large amounts of K or Vi antigen to resist the bactericidal activity of antibodies to the O somatic antigen (Nagington, 1956; Glynn and Howard, 1970). Moreover, there is no evidence for the presence of K type antigens on subcultured gonococci. The unconfirmed K antigen reported by Deacon et al. (1959, 1961) is clearly different to the postulated protective factor in that it can be induced by growth in vitro and appears to be unrelated to virulence (Kellogg et al., 1963). Nair and Chaco (1967) claimed to have shown that gonococci growing in the host possess an antigenic factor which is 'responsible' for gonococcal virulence and is absent on subcultured organisms. Because antigens derived from subcultured gonococci were used to detect antibodies which were produced against the postulated 'in vivo antigen' such an interpretation is difficult to accept.

The relative importance of serum killing as a host defence mechanism is so far unclear (Rosenberg, 1965). Nevertheless, patients with complement or immunoglobulin deficiencies generally show increased susceptibility to bacterial infections (Franglen, 1970; British Medical Journal, Editorial, 1970) although these factors are also essential for opsonising bacterial pathogens. It seems likely that resistance to serum killing must be an essential attribute for gonococci which are able to persist in the inflammatory exudate despite the presence of immunoglobulins and complement.

Because gonococcal virulence is also rapidly lost on subculture (Mahoney et al., 1946; Kellogg et al., 1963) it is likely that, for the first time, resistance to serum killing provides a parameter of gonococcal virulence which could be causally related to their pathogenicity. The problem is, therefore, what is the nature of this change to avirulence and serum sensitivity which occurs on subculturing gonococci?

The rapidity with which gonococci become serum sensitive on subculture is illustrated by the fact that even 18 hr. 0.25 - 0.5Ø mm microcolonies of gonococci in primary cultures were more susceptible to killing than the parent organisms which had been grown in the host. It is probable that the alterations involved are phenotypically induced, since it is difficult to believe that genetic mutations resulting in both serum sensitivity and an increased growth rate in vitro would occur with sufficient frequency. It is proposed to test this postulate by examining the sensitivity to serum of gonococci in urethral pus which have been cultured for various times on cellulose acetate filters. If the change to serum sensitivity is due to a genetic mutation the microcolonies would contain organisms of the original genotype which would continue to grow after exposure to serum. On the other hand if sensitivity to serum killing is due to a phenotypic change, all the organisms would become serum sensitive and fail to regrow after exposure to serum.

There are several examples in the literature of alterations, probably phenotypic in origin, which have resulted in a loss of virulence or an increased susceptibility of bacteria to host defence mechanisms (Meynell, 1961). Adlam et al. (1970 a and b) found that staphylococci grown in

the rabbit thorax showed an increased resistance to serum killing and phagocytosis by polymorphs. Cavanaugh and Randall (1959) found that S-type Pasteurella pestis were readily phagocytosed and killed by polymorphs. However, after cultivation in guinea pig monocytes, they became encapsulated and were then able to resist phagocytosis by polymorphs. The reversion of a phenotypic change has been demonstrated by Smith and Fitzgeorge (1964). They found that Brucella abortus recovered from infected bovine placental tissue had an increased ability to survive intracellular destruction compared with the same strain cultured on laboratory media. This was associated with a cell wall material which inhibited the intracellular destruction of avirulent Br. abortus and was not produced on subculture unless bovine foetal fluid was first added to the medium (Smith, 1968).

The importance of whether gonococci become avirulent and serum sensitive as a result of a phenotypically or a genotypically induced change is that a phenotypically induced alteration occurring on subculture should be reversible. The virulence of gonococci was related by Kellogg et al. (1963, 1968) with a genetic change associated with their colonial morphology. For reasons considered in chapter 1 this view is not entirely consistent with their data. Moreover, in this study no evidence was obtained of a correlation between colonial type and sensitivity to killing by a panel of normal human sera. Both the virulent type 1 and avirulent type 4 colonial forms of strain F62 which originated from Kellogg's laboratory were found to be highly sensitive to killing by normal or immune sera. Similarly, Walsh et al. (1963) associated the virulence

of gonococci with their ability to grow in the 8 day embryonated hen's egg. However, the present results showed that both a freshly isolated and an old laboratory strain of gonococcus grew in the 8 day egg with equal facility. Neither of these methods appears to be satisfactory for maintaining the virulence of gonococci. It seemed possible that growth within an animal might provide a nearer approximation to the environment of gonococci infecting the host. However, attempts to produce an experimental infection in mice which had been immunosuppressed with cortisol or methatrexate were unsuccessful. This was not because the mouse was nutritionally inadequate as a host because subcultured gonococci could grow in the mouse peritoneum when enclosed in a cellophane (Visking) or cellulose acetate (Millipore 0.45μ) sac. After intraperitoneal injection of gonococci suspended in haemoglobin or mucin, large members of the organisms were recovered from the blood stream. It seems likely that as for meningococci (Njoku-Obi, 1966) large numbers of gonococci are rapidly cleared from the blood by the reticuloendothelial system. The finding that gonococci grown in the mouse within a cellophane sac were highly sensitive to serum killing suggested that it was improbable that virulent gonococci could be maintained in this way. However, the problem of maintaining gonococci in urethral exudate for virulence studies was overcome by developing a technique for preserving them in liquid nitrogen. The high recovery rate of virtually 100% obtained by this means eliminated the possibility of atypical gonococci being selected by the preservation technique and compares very favourably with the 5-30% recovery rates previously obtained (Brookes and Hedén, 1966; Reyn, 1968).

In view of the recent promise of a meningococcal vaccine (Finland, 1970; Artenstein et al., 1970) and the antigenic and taxonomic relatedness of the gonococcus and meningococcus (Wilson, 1956; Kingsbury, 1967) it is of interest to compare the pathogenesis of their respective infections. In a prospective study on 14,744 new recruits, it was shown that systemic meningococcal infections only occurred in individuals lacking group specific meningococcal antibodies detectable by a bactericidal test, thereby explaining the age group incidence of meningitis (Goldschneider et al., 1969a). This confirmed and extended the earlier work of Hobbs et al. (1967) who showed in a series of patients with a familial susceptibility to systemic meningococcal infections that there was a correlated familial deficiency in bactericidal antibody, which in these particular patients was due to an IgM deficiency.

Immunisation of recruits with a high molecular weight preparation of the group C polysaccharide (Gotschlich et al., 1969a) which can be considered to be analagous to the K antigens of E.coli resulted in the development of group specific bactericidal antibodies (Gotschlich et al., 1969b). The mean nasopharyngeal carriage rate of the homologous group of meningococci in immunised recruits was reduced from 50% to 20% (Gotschlich et al., 1969c). Therefore there is good evidence for the protective role of humoral antibodies in systemic meningococcal infection. This work is supported by the epidemiology of meningitis, which is quite different from that of gonorrhoea. Thus in both meningococcal and gonococcal infections there is a high carrier rate. However, in meningitis unlike gonorrhoea there must be considerable natural resistance since the

morbidity is estimated at only 0.25% of the exposed population (Brown and Condit, 1965). Unlike gonorrhoea, meningitis is most prevalent in early infancy and childhood. In the United States epidemic meningococcal disease occurs in approximately 10 yr. cycles, suggesting that it requires an immunologically virgin population for its expression. In gonococci no capsular antigens comparable to the group specific meningococcal polysaccharides have been demonstrated. As shown in this work most individuals possess natural bactericidal antibodies to gonococci but there is little natural resistance to the disease and the frequency of multiple infections (Watt, 1958) suggests that no significant immunity follows gonococcal infection. There is therefore no evidence for a comparable protective role of humoral antibody in gonorrhoea. Such a role may be possible, however, in the relatively rare cases of systemic gonococcal infection, whose pathogenesis may be similar to that of systemic meningitis.

The finding of Goldschneider and his colleagues that the number of cases of systemic meningococcal disease was only 20% of the number of adults lacking group specific antibody suggests that lack of natural antibody detectable with a bactericidal test is not the sole determinant of infection. Other factors could include a deficiency in secretory IgA, nasopharyngeal abnormalities, the unknown factors contributing to the virulence of the organism and antecedent or concurrent viral infection. The demonstration in this work that virulent gonococci are protected from natural or immune antibodies as detected by the bactericidal reaction, supports the idea that in gonorrhoea it is the virulence of the organism,

rather than the host's antibody spectrum, which is of relatively greater importance. Whether antibodies raised against this protective factor would kill gonococci growing in the host remains to be seen. However, the ineffectiveness of gonococcal vaccines may be explained by the failure to maintain this factor.

APPENDIX 1.

Technique for preparing gonococcal lipopolysaccharide.

Reference

Maeland, J.A. (1968) Acta path. microbiol. scand., 73, 413-422.

Required

90% w/v Phenol solution (Caution).
Acetone.
Sodium Chloride.

Method

1) Prepare bulk growth of gonococci on plates or in Roux bottles.

2) Suspend in saline. Check purity of suspension.

3) Wash 3 times in saline. Determine yield of gonococci in grams wet weight.

4) Add 4 ml saline to packed gonococci for each gram wet weight.

5) Homogenise suspension with Waring Blender to break up clumps.

6) Add an equal volume of 90% w/v phenol. Extract with stirring for 20 min. at 37°C.

7) Cool. Centrifuge at 2,000g for 1 hr. at 4°C.

8) Remove and retain upper aqueous phase and measure its volume. The middle layer should consist of bacterial debris and the lower layer of phenol.

9) Replace aqueous phase with an equal volume of saline. Repeat steps 5, 6 and 7.

10) Centrifuge the combined aqueous phases at 6,000g for 30 min. to remove remaining insoluble debris.

11) Remove phenol by dialysis for 72 hr. against running tap water and frequent changes of distilled water.

12) Measure volume. Cool to 4°C. Add 3 times volume of cooled acetone and sufficient sodium chloride to induce precipitation. Allow to stand for 2 hr. at 4°C.

APPENDIX 1 (cont'd)

13) Collect the white precipitate by centrifugation. Redissolve in minimum volume of saline. Dialyse for 24 hr. against distilled water to remove traces of acetone.

14) Centrifuge 2 hr. at 105,000g under refrigeration. Redissolve brownish refractile pellet of lipopolysaccharide in minimum volume of water.

15) Repeat step 14 twice more to further purify lipopolysaccharide.

16) Lyophilise in weighed ampoule to determine yield of purified lipopolysaccharide in dry weight. Store at 4°C.

17) Determine the UV spectrum of a solution of 1mg/ml of the finally purified lipopolysaccharide. Should be free of absorption peaks at 260 and 280nm.

APPENDIX 2.

Glutaraldehyde fixation of red cells.

Reference

Bing, D.H., Weyand J.G.M. and Stavitsky, A.B. (1967) Proc. Soc. exp. Biol. Med., 124, 1166-1170.

Required

Washed Red Cells.
0.15 M Na_2HPO_4. Adjust to pH 8.2.
0.15 M NaCl.
25% Glutaraldehyde solution (Eastman Kodak).

Method

1) Make up the following salts solution: 1 vol. of 0.15 M Na_2HPO_4 pH 8.2 with 9 vols 0.15 M NaCl and 5 vols distilled water. Mix.

2) Add 25% glutaraldehyde to salts solution to a final concentration of 1% v/v.

3) Chill to $4^{\circ}C$.

4) Add washed packed red cells to glutaraldehyde-salts solution to a final concentration of 2% v/v.

5) Incubate with occasional stirring for 30 min. at $4^{\circ}C$.

6) Recover cells by centrifugation.

7) Wash 5 times with 0.15 M NaCl. Vol. of wash must be at least 10 times volume of packed cells.

8) Wash 5 times with distilled water. Cells should not lyse but become progressively browner.

9) Make up to approximately 25% v/v suspension in distilled water.

10) Check packed cell volume with a Haematocrit centrifuge.

11) Preserve with a final concentration of 1:10,000 merthiolate. Finally adjust cell suspension to 30% v/v.

12) Store at $4^{\circ}C$.

APPENDIX 2 (cont'd)

Sensitisation

Adjust suspension to 2½% v/v, and sensitise as for normal cells. After washing sensitised cells may be preserved with 1:10,000 merthiolate and stored at 4°C as a 2% v/v suspension.

Adlam, C., Pearce, J.H. and Smith, H. (1970a). Virulence mechanisms of staphylococci grown in vivo and in vitro. J. Med. Microbiol. 3, 147-155.

Adlam, C., Pearce, J.H. and Smith, H. (1970b). The interaction of staphylococci grown in vivo and in vitro with polymorphonuclear leucocytes. J. Med. Microbiol., 3, 157-163.

Amies, C.R. and Garabedian, M. (1967). An easily prepared selective medium for the cultivation of Neisseria gonorrhoeae. Br. J. vener. Dis., 43, 137-139.

Artenstein, M.S., Gold, R., Zimmerly, J.G., Wyle, F.A., Branche, W.C. and Harkins, C. (1970). Cutaneous reactions and antibody response to meningococcal group C polysaccharide vaccines in man. J. infect. Dis., 121, 372-377.

Atkin, E.E. (1925). The significance of serological types of gonococcus. Br. J. exp. Path., 6, 235-246.

Ayyangar, R.M.C. (1970). Venereal diseases in animals. Indian J. Derm. Vener., 36, 15-22.

Berger, U. (1966). Neue elektivnährböden für Neisseria meningitidis und Neisseria gonorrhoeae. Z med. Mikrobiol. Immun. 152, 169-172.

Bernstein, L.H.T. (1941). Capsulation of Neisseria gonorrhoeae. Proc. Soc. exp. Biol. Med., 46, 700-703.

Bing, D.H., Weyand, J.G.M. and Stavitsky, A.B. (1967). Haemagglutination with aldehyde-fixed erythrocytes for assay of antigens and antibodies. Proc. Soc. exp. Biol. Med., 124, 1166-1170.

Blanden, R.V., Mackaness, G.B. and Collins, F.M. (1966). Mechanisms of acquired resistance in mouse typhoid. J. exp. Med., 124, 585-600.

Boor, A.K. and Miller, C.P. (1944). A carbohydrate-lipid fraction of gonococcus and meningococcus. J. infect. Dis., 75, 47-57.

British Medical Journal, Editorial (1970). Recurrent infection and deficiency of complement. Br. med. J. ii, 744.

Brookes, R. and Hedén, C.-G. (1966). The preservation of N.gonorrhoeae by controlled-rate freezing followed by storage in liquid nitrogen. Biotech. Bioeng., 7, 315-317.

Brown, J.W. and Condit, P.K. (1965). Meningococcal infections. Fort Ord and California. Calif. Med., 102, 171-180.

Bullen, J.J., Leigh, L.C. and Rogers, H.J. (1968). The effect of iron compounds on the virulence of Escherichia coli for guinea-pigs. Immunology, 15, 581-588.

Carpenter, P.L. (1965). Immunology and Serology. 2nd ed., p. 110. pub.Saunders and Co., Philadelphia.

Casper, W.A. (1937). The preparation of the type-specific carbohydrates of gonococci. J. Immun., 32, 421-439.

Casper, W.A. (1941). The biochemistry of the gonococcus and its practical importance. J. vener. Dis. Inform., 22, 119-123.

Catterall, R.D. (1969). Current problems in urethritis. Medical Research Council conference, 9th December, 1969, London.

Cavanaugh, D.C. and Randall, R. (1959). The role of multiplication of Pasteurella pestis in mononuclear phagocytes in the pathogenesis of flea-borne plague. J. Immun., 83, 348-363.

Chacko, C.W. and Nair, G.M. (1966). A new convenient egg-enriched medium for the cultural diagnosis of gonorrhoea. Indian J. Derm. Vener., 32, 41-51. WHO/VDT/RES/GON 66.12

Chacko, C.W. and Nair, G.M. (1969). Sero-diagnosis of gonorrhoea with a microprecipitin test using a lipopolysaccharide antigen from N.gonorrhoeae. Br. J. vener. Dis., 45, 33-39.

Chanarin, I. (1954). An investigation of Neisseria gonorrhoeae by a red cell sensitization technique. J. Hyg., Camb., 52, 425-443.

Chatelain, R. (1963). A propos de l'utilisation du glutamate de sodium dans la lyophilisation des bacteries. Annls. Inst. Pasteur, Paris, 105, 111-117.

Cohen, I.R. (1967). Natural and immune human antibodies reactive with antigens of virulent Neisseria gonorrhoeae: immunoglobulins G, M and A. J. Bact., 94, 141-148.

Cohen, I.R., Norins, L.C. and Julian, A.J. (1967). Competition between, and effectiveness of, IgG and IgM antibodies in indirect fluorescent antibody and other tests. J. Immun., 98, 143-149.

Cohen, I.R., Kellogg, D.S. and Norins, L.C. (1969). Serum antibody response in experimental human gonorrhoea. Immunoglobulins G, A and M. Br. J. vener. Dis., 45, 325-327.

Daguillard, F. and Edsall, G. (1968). The agglutinating and bactericidal activity of IgM and IgG antibodies to the 9 and 12 factors of Salmonella typhi O 901. J. Immun., 100, 1112-1120.

Danielsson, D. (1965). The demonstration of N.gonorrhoeae with the aid of fluorescent antibodies. 3 Studies by immunofluorescence and double diffusion-in-gel technique on the antigenic relationship between strains of N.gonorrhoeae. Acta path. microbiol. scand., 64, 243-266.

Danielsson, D.G., Schmale, J.D., Peacock, W.L. and Thayer, J.D. (1969). Antigens of Neisseria gonorrhoeae: characterization by gel filtration, complement fixation, and agar-gel diffusion of antigens of gonococcal protoplasm. J. Bact., 97, 1012-1017.

Danielsson, D., Schmale, J.D. and Lee, Linda (1970). U.S.A.: Studies of the immune response in gonococcal infections with various serological techniques. Acta path. microbiol. scand., 78B, 267.

Davies, D.A.L., Crumpton, M.J., Macpherson, I.A. and Hutchison, Ann M. (1958). The adsorption of bacterial polysaccharides by erythrocytes. Immunology, 1, 157-171.

Davis, R.H. and Pelczar, M.J. (1967). Cultivation of gonococci in tissue culture. Bact. Proc., (1967) 78.

Deacon, W.E. (1961). Fluorescent antibody methods for Neisseria gonorrhoeae identification. Bull. Wld Hlth Org., 24, 349-355.

Deacon, W.E., Peacock, W.L., Freeman, Elizabeth M. and Harris, A. (1959). Identification of Neisseria gonorrhoeae by means of fluorescent antibodies. Proc. Soc. exp. Biol. Med., 101, 322-325.

Felix, A. and Olitzki, L. (1926). The qualitative receptor analysis. II. Bactericidal serum action and qualitative receptor analysis. J. Immun., 11, 31-80.

Finegold, D.S. (1969). The serum bactericidal reaction IV. Phenotypic conversion of Escherichia coli from serum-resistance to serum-sensitivity by diphenylamine. J. infect. Dis., 120, 437-444.

Finland, M. (1970). Revival of antibacterial immunization: Meningococcal vaccines prove promising. J. infect. Dis., 121, 445-448.

Fitz-James, P. (1964). Thin sections of dividing Neisseria gonorrhoeae. J. Bact., 87, 1477-1482.

Franglen, G. (1970). Immune globulin deficiencies. Br. J. hosp. Med., 3, 651-660.

Fry, R.M. (1951). The Influence of the Suspending Fluid on the Survival of Bacteria after Drying. In Harris, R.J.C. (ed.) Freezing and Drying, 1 ed., pp 107-115, pub. Institute of Biology, London, 1952.

Gavrilescu, M., Lazăr, M., Porojan, I. and Cîrciumărescu, T. (1966). Long-term cultivation of N.gonorrhoeae in tissue culture. Br. J. vener. Dis. 42, 171-174.

Gavrilescu, M. and Lazăr, M. (1968). Etudes sur la methode de cultivation de N.gonorrhoeae dans les cultures tissulaires. Actas dermosifilogr., 59, 429-442.

Gerhardt, P. and Hedén, C.-G. (1960). Concentrated culture of gonococci in clear liquid medium. Proc. Soc. exp. Biol. Med., 105, 49-51.

Glynn, A.A. and Howard, C.J. (1970). The sensitivity to complement of strains of Escherichia coli related to their K antigens. Immunology, 18, 331-346.

Gold, R. and Wyle, F.A. (1970). New classification of Neisseria meningitidis by means of bactericidal reactions. Infect. Immun., 1, 479-484.

Goldschneider, I., Gotschlich, E.C. and Artenstein, M.S. (1969a). Human immunity to the meningococcus. I. The role of humoral antibodies. J. exp. Med., 129, 1307-1326.

Goldschneider, I., Gotschlich, E.C. and Artenstein, M.S. (1969b). Human immunity to the meningococcus. II. Development of natural immunity. J. exp. Med., 129, 1327-1348.

Gotschlich, E.C., Liu, T.Y., and Artenstein, M.S. (1969a). Human immunity to the meningococcus. III. Preparation and immunochemical properties of the group A, group B, and group C meningococcal polysaccharides. J. exp. Med., 129, 1349-1365.

Gotschlich, E.C., Goldschneider, I. and Artenstein, M.S. (1969b). Human immunity to the meningococcus. IV. Immunogenieity of group A and group C meningococcal polysaccharides in human volunteers. J. exp. Med., 129, 1367-1384.

Gotschlich, E.C., Goldschneider, I. and Artenstein, M.S. (1969c). Human immunity to the meningococcus. V. The effect of immunization with meningococcal group C polysaccharide on the carrier state. J. exp. Med., 129, 1385-1395.

Greaves, R.I.N. (1960). Some Factors which Influence the Stability of Freeze-Dried Cultures. In Parkes, A.S. and Smith, A.U. (eds.) Recent Research in Freezing and Drying, 1st ed., pp 203-215, pub. Blackwell, Oxford.

Harkness, A.H. (1948). The pathology of gonorrhoea. Br. J. vener. Dis., 24, 137-147.

Hill, Justina H. (1943). Experimental infection with Neisseria gonorrhoeae. I. Human inoculations. Am. J. Syph. Gonorrhea vener. Dis., 27, 733-771.

Hill, Justina H. (1944). Experimental infection with Neisseria gonorrhoeae. II. Animal inoculations. Am. J. Syph. Gonorrhea vener. Dis., 28, 334-378, 471-510.

Hill, Justina, H., Huffer, Virginia and Nell, Ellen (1945). The problem of infection of animal mucosae with Neisseria gonorrhoeae. I. Modification of host resistance. Am. J. Syph. Gonorrhea vener. Dis., 29, 281-302.

Hill, Justina H. and Pitts, A.C. (1939). The growth of Neisseria gonorrhoeae on the chorio-allantoic membrane of the chick embryo. J. Urol., 41, 81-83.

Hobbs, J.R., Milner, R.D.G. and Watt, P.J. (1967). Gamma-M deficiency predisposing to meningococcal septicaemia. Br. med. J., iv, 583-586.

Holmes, K.K., Johnson, D.W. and Trostle, H.J. (1970). An estimate of the risk of men acquiring gonorrhoea by sexual contact with infected females. Am. J. Epidem., 91, 170-174.

Jenkin, C.R. and Rowley, D. (1963). Basis for immunity to typhoid in mice and the question of 'cellular immunity'. Bact. Rev., 27, 391-404.

Jutila, J.W. and Reed, N.D. (1968). Wasting disease induced with cortisol acetate. II. Bacteriologic studies. J. Immun., 100, 675-681.

Kabat, E.A. and Mayer, M.M. (1961). Experimental Immunochemistry. 2nd ed., p.135. pub. Thomas, Springfield, Illinois.

Karthigasu, K., Reade, P.C. and Jenkin, C.R. (1965). The functional development of the reticulo-endothelial system. III. The bactericidal capacity of fixed macrophages of foetal and neonatal chicks and rats. Immunology, 9, 67-73.

Keiser, H., Ruben, F.L., Wolinsky, E. and Kushner, I. (1968). Clinical forms of gonococcal arthritis. New Engl. J. Med., 279, 234-240.

Kellogg, D.S., Peacock, W.L., Deacon, W.E., Brown, L. and Pirkle, C.I. (1963). Neisseria gonorrhoeae. I. Virulence genetically linked to clonal variation. J. Bact., 85, 1274-1279.

Kellogg, D.S., Cohen, I.R., Norins, L.C., Schroeter, A.I. and Reising, G. (1968). Neisseria gonorrhoeae. II. Colonial variation and pathogenieity during 35 months in vitro. J. Bact., 96, 596-605.

Kellogg, D.S. and Thayer, J.D. (1969). Virulence of gonococci. A. Rev. Med., 20, 323-328.

Kenny, C.P. and Sparkes, B.G. (1968). Inhibitor of bacterial growth released by human cells in culture. Science, N.Y., 161, 1344-1345.

Kingsbury, D.T. (1967). Deoxyribonucleic acid homologies among species of the genus Neisseria. J. Bact., 94, 870-874.

Kocka, F.E. and Bretz, H.W. (1969). Survival of Escherichia coli after storage in various frozen menstrua. Antonie van Leeuwenhoek, 35, 65-76.

La Brec, E.H., Schneider, H., Magnani T.J. and Formal, S.B. (1964). Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentry. J. Bact., 88, 1503-1518.

Labzoffsky, N.A. and Kelen, A.E. (1961). New complement fixing antigen for serodiagnosis of gonorrhoea. Can. J. Microbiol., 7, 715-723.

Lee, Linda and Schmale, J.D. (1970). Identification of a gonococcal antigen important in the human immune response. Infect. Immun., 1, 207-208.

Lind, Inga (1967). Identification of Neisseria gonorrhoeae by means of a fluorescent antibody technique. Acta path. microbiol. scand., 70, 613-629.

Lüderitz, O., Jann, K. and Wheat, R. (1968). Somatic and Capsular Antigens of Gram Negative Bacteria. In Florkin, M. and Stotz, E.H. (eds.) Comprehensive Biochemistry, Vol. 26A, 1st ed., pp. 105-228, pub. Elsevier, Amsterdam.

Maeland, J.A. (1966). Antibodies in human sera against antigens in gonococci, demonstrated by a passive haemolysis test. Acta path. microbiol. scand., 67, 102-110.

Maeland, J.A. (1967). Serological properties of antisera to Neisseria gonorrhoeae antigens. Acta path. microbiol. scand., 69, 145-155.

Maeland, J.A. (1968). Antigenic properties of various preparations of Neisseria gonorrhoeae endotoxin. Acta path. microbiol. scand., 73, 413-422.

Maeland, J.A. (1969a). Antigenic determinants of aqueous ether extracted endotoxin from Neisseria gonorrhoeae. Acta path. microbiol. scand., 76, 475-483.

Maeland, J.A. (1969b). Immunochemical characterization of aqueous ether extracted endotoxin from Neisseria gonorrhoeae. Acta path. microbiol. scand., 76, 484-492.

Maeland, J.A. (1969c). Serological properties of aqueous ether extracted endotoxin from Neisseria gonorrhoeae. Acta path microbiol. scand., 77, 495-504.

Maeland, J.A. (1969d). Serological cross-reactions of aqueous ether extracted endotoxin from Neisseria gonorrhoeae strains. Acta path. microbiol. scand., 77, 505-517.

Mahoney, J.F., van Slyke, C.J., Cutler, J.C. and Blum, H.L. (1946). Experimental gonococcic urethritis in human volunteers. Amer. J. Syph. Gonorrhea vener. Dis., 30, 1-39.

Martin, J.E., Peacock, W.L., Reising G., Kellogg, D.S., Ribi, E. and Thayer, J.D. (1969). Preparation of cell walls and protoplasm of Neisseria with the Ribi cell fractionator. J. Bact., 97, 1009-1011.

McCarty, M. (1958). Further studies on the chemical basis for serological specificity of Group A streptococcal carbohydrate. J. exp. Med., 108, 311-323.

Meynell, G.G. (1961). Phenotypic Variation and Bacterial Infection. In Meynell, G.G. and Gooder, H. (eds.) Microbial Reaction to Environment, pp. 174-195. 11th Symp. Soc. gen. Microbiol., Cambridge, 1961.

Miller, C.P. (1944). The enhancement of virulence of the gonococcus for the mouse. Am. J. Syph. Gonorrhea vener. Dis., 28, 620-626.

Miller, C.P., Drell, M.J., Moeller, Velma and Bohnhoff, Marjorie (1945). Experimental gonococcal infection of the rabbits' eyes. I. Method of production. J. infect. Dis., 77, 193-200.

Milner, K.C. and Finkelstein, R.A. (1966). Bioassay of endotoxin: Correlation between pyrogenicity for rabbits and lethality for chick embryos. J. infect. Dis., 116, 529-536.

Nagington, J. (1956). The sensitivity of Salmonella typhi to the bactericidal action of antibody. Br. J. exp. Path., 37, 397-405.

Nair, G.M. and Chacko, C.W. (1966a). A biologically active substance from N.gonorrhoeae grown in embryonated chick eggs. WHO/VDT/RES/GON 66.14.

Nair, G.M. and Chacko, C.W. (1966b). Kinetics of a microprecipitin test in the sero-diagnosis of gonorrhoea. WHO/VDT/RES/GON 66.15.

Nair, G.M. and Chacko, C.W. (1967). An antigenic fraction in N.gonorrhoeae responsible for the virulence of the organism. Indian J. Derm. Vener., 33, 53-64.

Neter, E., Westphal, O., Lüderitz, O., Gorzynski, E.A. and Eichenberger, E. (1956). Studies of enterobacterial lipopolysaccharides. Effects of heat and chemicals on erythrocyte-modifying, antigenic, toxic and pyrogenic properties. J. Immun., 76, 377-385.

Njoku-Obi, A.N.U. (1966). Immunity in experimental meningococcal infection. W. Afr. med. J., 15, 109-114.

Obayashi, Y., Ota, S. and Arai, S. (1961). Some factors affecting preservability of freeze-dried bacteria. J. Hyg., Camb., 59, 77-91.

Ogawa, H., Nakamura, A., Nakaya, R., Katsutoshi, M., Honjo, S., Takasaka, M., Fujiwara, T. and Imaizumi, K. (1967). Virulence and epithelial cell invasiveness of dysentry bacilli. Jap. J. med. Sci. Biol., 20, 315-328.

Ovčinnikov, N.M. (1963). An appraisal of the fluorescent antibody method in gonorrhoea. Bull. Wld Hlth Org., 29, 781-788.

Ovčinnikov, N.M. and Delektorskij, V.V. (1966). The structure of the gonococcus as seen in study of ultrathin sections under the electron microscope. WHO/VDT/RES/GON. 66.16.

Pariser, H., Farmer, A.D. and Marino, A.F. (1964). Asymptomatic gonorrhoea in the male. Sth. med. J., Nashville, 57, 688-690.

Peacock, W.L. and Schmale, J.D. (1969). Toxic constituents of Neisseria gonorrhoeae. Nature, Lond., 221, 760-761.

Pierce, Cynthia H., Dubos, R.J. and Schaefer, W.B. (1953). Multiplication and survival of tubercle bacilli in the organs of mice. J. exp. Med., 97, 189-205.

Potužnik, V. and Hausner, O. (1969). A selective medium with lincomycin and colistin used in isolation of N.gonorrhoeae and N.meningitidis. J. Hyg. Epidem. Microbiol. Immun., 13, 366-373.

Price, J.N.O. (1930). The gonococcal complement fixation test: Improvements in technique. J. Path. Bact., 33, 493-495.

Rees, Elisabeth and Annels, E.H. (1969). Gonococcal salpingitis. Br. J. vener. Dis., 45, 205-215.

Reising, R. and Kellogg, D.S. (1965). Detection of gonococcal antibody. Proc. Soc. exp. Biol. Med., 120, 660-663.

Reising, G., Schmale, J.D., Danielsson, D.G. and Thayer, J.D. (1969). Reactivity of two selected antigens of Neisseria gonorrhoeae. Appl. Microbiol., 18, 337-339.

Reyn, Alice (1949a). Serological studies on gonococci. I. Technique. Gono-reaction of "normal" rabbits. Serological relation between gonococci and pasteurellae. Acta path. microbiol. scand., 26, 51-70.

Reyn, Alice (1949b). Serological studies on gonococci. II. Cross absorption experiments and factor serum determinations. Acta path. microbiol. scand., 26, 234-251.

Reyn, Alice (1949c). Serological studies on gonococci. III. Thermostability and biochemical nature of the gonococcus antigens. Durability of the factor sera. Discussion. Acta path. microbiol. scand., 26, 252-268.

Reyn, Alice, Murray, R.G.E. and Birch-Andersen, A. (1963). Electron microscopical study of a strain of Neisseria gonorrhoeae. WHO/VDT/RES/GON 63.1.

Reyn, Alice (1968). Storage and maintenance of WHO international reference strains of Neisseria gonorrhoeae for the determination of sensitivity to penicillin. WHO/VDT/RES/GON 68.22.

Robbins, J.B., Kenny, Kathryn and Suter, E. (1965). The isolation and biological activities of rabbit gamma M and gamma G - anti - Salmonella typhimurium antibodies. J. exp. Med., 122, 385-402.

Rosenberg, L.T. (1965). Complement. A. Rev. Microbiol., 19, 285-300.

Schaedler, R.W., Dubos, R. and Costello, R. (1965). The development of the bacterial flora in the gastrointestinal tract of mice. J. exp. Med., 122, 59-66.

Scherp, H.W. (1955). Neisseria and neisserial infections. A. Rev. Microbiol., 9, 319-334.

Schmale, J.D., Danielsson, D.G., Smith, J.F., Lee, Linda and Peacock, W.L. (1969). Isolation of an antigen of Neisseria gonorrhoeae involved in the human immune response to gonococcal infection. J. Bact., 99, 469-471.

Smith, H. (1968). Biochemical challenge of microbial pathogenicity. Bact. Rev., 32, 164-184.

Smith, H. and Fitzgeorge, R.B. (1964). The chemical basis of the virulence of Brucella abortus. V. The basis of intracellular survival and growth in bovine phagocytes. Br. J. exp. Path., 45, 174-186.

Sparling, P.F. and Yobs, Anne R. (1967). Colonial morphology of Neisseria gonorrhoeae isolated from males and females. J. Bact., 93, No. 1, 513.

Spink, W.W. and Keefer, C.S. (1937). Studies of gonococcal infection. I. A study of the mode of destruction of the gonococcus in vitro. J. clin. Invest., 16, 169-176.

Stokinger, H.E., Ackerman, Helen and Carpenter, C.M. (1944a). Studies on the gonococcus. I. Constituents of the cell. J. Bact., 47, 129-139.

Stokinger, H.E., Ackerman, Helen and Carpenter, C.M. (1944b). Studies on the gonococcus. II. Properties of an antigenic fraction isolated from cell-free gonococcal broth supernatants. J. Bact., 47, 141-147.

Stokinger, H.E., Carpenter, C.M. and Plack, Jane (1944c). Studies on the gonococcus. III. Quantitative agglutinative reactions of the Neisseria with special reference to Neisseria gonorrhoeae. J. Bact., 47, 149-157.

Szent, Györgyi A., Együd, L.G. and McLaughlin, Jane A. (1967). Keto-aldehydes and cell division. Science, N.Y., 155, 539-541.

Tauber, H. and Russell, H. (1961). Biochemistry of Neisseria gonorrhoeae endotoxin. Bull. Wld Hlth Org., 24, 385-386.

Thayer, J.D. and Martin, J.E. (1966). Improved medium selective for cultivation of N.gonorrhoeae and N.meningitidis. U.S. Publ. Hlth Rep., 81, 559-562.

Thomas, J.C. and Mennie, A.T. (1950). Bacterial polysaccharides in the diagnosis of infections. The polysaccharide lysis test. Lancet, ii, 745-746.

Tripodi, D. and Nowotny, A. (1966). Relation of structure to function in bacterial O-antigens. V. Nature of active sites in endotoxic lipopolysaccharides of Serratia marcescens. Ann. N.Y. Acad. Sci., 133, 604-621.

Ueda, K., Diena, B.B. and Greenberg, L. (1969). The chick embryo neutralization test in the assay of meningococcal antibody. 1. Infection of the embryo with Neisseria meningitidis. Bull. Wld Hlth Org., 40, 235-240.

Uroma, E. (1943). Untersuchungen uber die Typenspezifitat der Gonokokkenpolysaccharide. Acta derm.-vener., Stockh., 23, Supplement IX, 1-74.

Wallace, R., Diena, B.B. and Greenberg, L. (1970). Bentonite flocculation test in the assay of Neisseria antibody. Bact. Proc. (1967) 110.

Walsh, M.J., Brown, B.C., Brown, L. and Pirkle, C.I. (1963). Use of the chick embryo in maintaining and restoring virulence of Neisseria gonorrhoeae. J. Bact., 86, 478-481.

Watt, L. (1958). Gonorrhoea in Manchester. Br. J. vener. Dis., 34, 9-13.

Watt, P.J. (1970). The fate of gonococci in polymorphonuclear leukocytes. J. Med. Microbiol. In Press.

Westphal, O., Lüderitz, O. and Bister, F. (1952). Uber die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturf., 7B, 148-155.

White, L.A. and Kellogg, D.S. (1965). Neisseria gonorrhoeae identification in direct smears by a fluorescent antibody-counterstain method. Appl. Microbiol., 13, 171-174.

WHO (1963). WHO expert committee on gonococcal infections. 1st report. Wld Hlth Organ. techn. Rep. Ser., 262, 1-72.

WHO (1965). Meeting on Neisseria research. 1st report. WHO/VDT/RES/GON 8.65. WHO/BD/MEN/1.65.

Willcox, R.R. (1969). Current problems in urethritis. Medical Research Council conference, 9th December, 1969, London.

Willoughby, D.A. and Giroud, J.P. (1969). The role of polymorphonuclear leucocytes in acute inflammation in agranulocytic rats. J. Path., 98, 53-60.

Wilson, J.F. (1954). A serological study of Neisseria gonorrhoeae. J. Path. Bact., 68, 495-510.

Wilson, J.F. (1956). A serological study of the meningococcus. J. Path. Bact., 72, 111-119.

Zeya, H.I. and Spitznagel, J.K. (1968). Arginine-rich proteins of polymorphonuclear leukocyte lysosomes. Antimicrobial specificity and biochemical heterogeneity. J. exp. Med., 127, 927-941.

Publications

Glynn, A.A. and Ward, M.E. (1970). Nature and heterogeneity of the antigens of Neisseria gonorrhoeae involved in the serum bactericidal reaction. Infect. Immun., 2, 162-168.

Ward, M.E., Watt, P.J., and Glynn, A.A. (1970). Gonococci in urethral exudates possess a virulence factor lost on subculture. Nature, Lond., 227, 382-384.

Ward, M.E. and Watt, P.J. (1970). The preservation of gonococci in liquid nitrogen. WHO/VDT/RES/GON 70.39. J. clin. Path. (in press).

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Nature and Heterogeneity of the Antigens of *Neisseria gonorrhoeae* Involved in the Serum Bactericidal Reaction

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Sixty strains of *Neisseria gonorrhoeae* have been classified into four main groups according to their resistance to killing by human complement together with either normal human or immune rabbit antibodies. The rabbit antisera had been raised against 10 of the strains tested. The normal human antibodies had probably been formed against cross-reacting organisms since they could be removed by absorption with *N. pharyngis sicca*, *N. pharyngis flavus*, *N. catarrhalis*, or *Escherichia coli*. Bactericidal antibodies could be absorbed from both normal and immune sera by *N. gonorrhoeae* which had been autoclaved or trypsinized, and by red cells coated with gonococcal lipopolysaccharides. The results suggest that the antigens involved in the bactericidal reaction are lipopolysaccharides of several distinct specificities. Since individual sera always reacted more widely in hemagglutination than in bactericidal tests, it is postulated that surface-blocking antigens may restrict access to the lipopolysaccharides in the intact organisms.

Ninety years after the discovery of gonococci by Neisser, knowledge of its antigenic structure remains rudimentary compared with what is known about streptococci or salmonellae. To a large extent this is due to the difficulties in growing *Neisseria gonorrhoeae* and to the absence of a satisfactory model animal infection or of any test for virulence. Early work on gonococcal antigens was summarized by Wilson and Miles (19). Their rather general conclusion was that there are two main types of *N. gonorrhoeae*, several intermediate types, a number of antigens, and a close antigenic relationship to *N. meningitidis*. More recent developments were the detection of antigens by means of the fluorescent antibody technique (5, 8), the use of hemagglutination techniques (3, 12), the study of types of antibody found (4), and the beginning of a diagnostic precipitin test (2). There have also been some chemical and serological analyses of gonococcal endotoxins (18, 13). Very promising results are appearing from the intensive study now being made of antigens from cell wall and cytoplasmic fractions of gonococci (14) and their reactions with patient's serum (6, 17). For the moment, however, gonococcal antigens remain largely symbols, potentially useful but of uncertain function and doubtful composition.

A more detailed knowledge of gonococcal antigens is essential for the future investigation of immune responses in gonorrhoea. It is now clear (2, 4) that antibodies develop even if immunity does not. A simple, reliable, serological test for early gonococcal infection would be invaluable for the investigation of female patients in whom direct culture is unreliable. A serological classification of *N. gonorrhoeae* would also be useful in epidemiological work.

The killing of *N. gonorrhoeae* by human serum seemed to us to provide a possible test system for detecting gonococcal antigens which could conceivably be of biological significance and could be used as the basis for later chemical investigation.

This paper reports the results of treating 60 strains of *N. gonorrhoeae* with normal human serum antibody or immune rabbit antibody in the presence of human complement. Some preliminary experiments were also made on the nature and specificity of the antigens involved, and the bactericidal results have been compared with those of hemagglutination tests using sheep red cells sensitized with gonococcal extracts.

MATERIALS AND METHODS

Strains of *N. gonorrhoeae* (G1, G3-G20, and G22-G60) were obtained from patients attending the

venereal diseases clinic of St. Mary's Hospital. Urethral or cervical exudates were first cultured on either chocolate-serum-agar or on the medium described by Amies and Garabedian (1) but with the antibiotics omitted. Organisms were identified by Gram staining, oxidase, and fermentation reactions, and were then preserved by lyophilization after the minimal number of subcultures required to ensure purity, usually one to two. A new ampoule was opened for each experiment. Strains G2 and G21, NCTC 8375, and NCTC 8448 were provided by the National Collection of Type Cultures, Colindale, as were *N. meningitidis* NCTC 8339, *N. flavescens* NCTC 8263, *N. pharyngis-sicca* NCTC 4591, *N. pharyngis-flavus* NCTC 4590, and *N. catarrhalis* NCTC 3622. *N. gonorrhoeae* strains 1a, 2c, 42981, 12954, 11413, and 44341 were kindly given to us by A. Reyn of the State Serum Institute, Copenhagen, and have already been described by her (15). *Escherichia coli* WF96 and *Pseudomonas aeruginosa* R 133 were from the culture collection at the Wright-Fleming Institute, St. Mary's Hospital.

Gonococci were incubated at 37 C in specially modified milk churns (16) under a manometrically adjusted atmosphere of 5% CO_2 in air in the presence of excess moisture.

Sera. Samples of normal human serum were obtained from nine adult male donors with no previous history of gonococcal infection and a negative gonococcal complement fixation test. A portion of the serum from eight of these donors was pooled, and serum from the ninth donor (MW) was used as a standard source of complement and normal antibody for the bactericidal test, except where otherwise indicated. All sera were stored at −60 C in 1-ml portions and were thawed once only. Serum MW had a titer of 52 complement H_{50} units/ml, and the pooled serum of 50 complement H_{50} units/ml when assayed by the method of Kabat and Mayer (11).

Rabbit antigonococcal sera were prepared against strains G 1, 2, 3, 4, 7, 22, 37, 38, 50, and 52 by intravenous injection of approximately 10^9 viable gonococci in saline on days 0 and 3. The rabbits were bled on day 17, and the sera were tested and stored at −20 C. Only rabbits whose normal serum showed no gonococcidal activity at a final concentration of 1:100 in the bactericidal assay were chosen for immunization.

Bactericidal test. The overnight growth of gonococci from an ampoule cultured on Amies and Garabedian medium was suspended in broth to a concentration corresponding to 4×10^8/ml. A 0.5-ml amount of this was used to inoculate the biphasic flask culture described by Gerhardt and Hedén (9) modified by leaving out the starch from the broth and reducing the volume. After 3 hr of incubation at 37 C, the liquid phase contained approximately 10^9 gonococci per ml in the early logarithmic stage of growth. These were diluted approximately 1,000 times in Dulbecco's complete balanced salt solution to give a concentration of viable organisms of about 10^6 per ml. This was sufficient to dilute out the anticomplementary effect of broth and made it possible to avoid washing the bacteria which had been found to reduce the viable count significantly. A 0.1-ml amount of a suspension containing approximately 10^5 organisms was added to tubes containing 0.25 ml of fresh or deep-frozen normal human serum (MW) and 0.1 ml of a 1:10 dilution in Dulbecco's solution of rabbit immune serum where appropriate; the volume was made up to 1.0 ml with Dulbecco's solution. The final concentration of complement, therefore, was approximately 13 complement H_{50} units per ml. The tubes were then incubated for 30 min at 37 C in a shaking water bath. Control tubes containing 0.25 ml of inactivated normal human serum with rabbit antibody, where appropriate, were set up in parallel. Samples (0.1 ml) were taken at 0 and 30 min, diluted immediately in Dextrose Starch Broth (Difco) without the starch, and spread on plates of Amies and Garabedian's medium to determine viable counts. Strains were defined as sensitive when the test tube showed a reduction of 90% (i.e., 1 log unit) or more in viable count in 30 min when compared with the control lacking complement. The frequency distribution of percentages of organisms killed in 210 tests of 60 strains is shown in Fig. 1.

All strains were first screened by using normal serum to provide complement and antibody. Resistant strains were retested with the addition to the system of rabbit immune serum.

Other bactericidal tests were carried out by using various normal and absorbed immune sera as sources of antibody. One to two milliliters of a 1:5 dilution of normal human serum or a 1:10 dilution of rabbit antiserum in Dulbecco's balanced salt solution was absorbed twice for 12 hr at 4 C with 10% (v/v) of packed bacteria in sterile tubes [3 by 0.5 inches (7.6 by 1.3 cm)]. All antisera and absorbed sera were checked for significant anticomplementary activity by the use of strain G2, which was sensitive to the bactericidal activity of the standard normal human serum alone. Specificity of absorption was checked by control tubes containing antibody absorbed with heterologous organisms.

Gonococcal extracts. Extracts were prepared from strains G1, G2, G22, G36, and G38 by the phenol-water method (12). The yield of deposit on ultracentrifugation of the water layer was 2 mg/g (wet

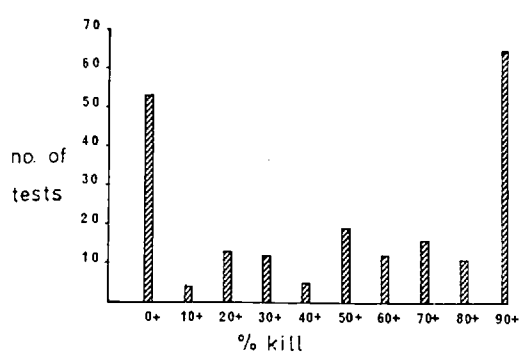


FIG. 1. *Frequency distribution of percentage of organisms killed in 210 bactericidal tests with Neisseria gonorrhoeae, using one normal human serum (MW) and four immune rabbit sera.*

weight) of bacteria. The ultraviolet spectra were similar to those reported by Maeland (12), and there were no absorption peaks at 260 and 280 nm. Preparations from G1 and G36 induced a biphase febrile response and a local Schwartzman reaction in rabbits. Although full chemical analysis has not yet been done, the method of preparation, the absence of detectable protein, the marked endotoxinlike activity (2 μg was pyrogenic), and the ability of alkali-treated material to sensitize red cells suggest that the active fractions concerned were Westphal-type lipopolysaccharides.

To sensitize sheep red cells, the lipopolysaccharide was treated with alkali (7), made up to 12.5 μg/ml in Dulbecco's solution and incubated with an equal volume of 2.5% red cells for 1 hr at 37 C. The cells were washed three times before use.

Hemagglutination was carried out in Perspex trays by using 0.1-ml volumes of twofold dilutions of antibody and 0.1 ml of 0.5% sensitized red cells.

Antisera were absorbed by mixing a 1:10 dilution of antiserum with 20% (v/v) packed sensitized red cells, mixing and keeping for 4 hr or overnight at 4 C. The cells were then removed by centrifugation, and the procedure was repeated four more times.

RESULTS

Serum sensitivity of strains of N. gonorrhoeae. The results of the bactericidal tests on strains of *N. gonorrhoeae* G1-G60 are shown in Fig. 2. There appear to be four main groups, defined by the antibodies necessary for killing by complement to occur.

Group I strains were killed in the presence of antibodies from normal human serum (serum MW).

Group II strains were killed when any of the immune anti-G1, -G2, -G3, -G4, -G37, or -G50 sera were present.

Group III strains were killed in the presence of anti-G37 or anti-G50 but not of anti-G1, -G2, -G3, or -G4 serum.

Group IV was resistant to the antisera just mentioned and also to anti-G7, -G22, -G38, and -G52. This group is of particular interest in that strains G22 and G52 were resistant to their homologous antisera. Strain G7 was also resistant to anti-G7 on our criteria, although there was a 30% kill. G38 is included in the group for the time being, although it was killed when anti-G38 was added.

Detailed examination of Fig. 2 shows several other strains which do not fit exactly into the groups described, e.g., G1 resists anti-G37 and anti-G50. G24 and G25 are slightly sensitive to normal human serum (MW) yet resist anti-G3 and anti-G4 or anti-G37. Exact classification must

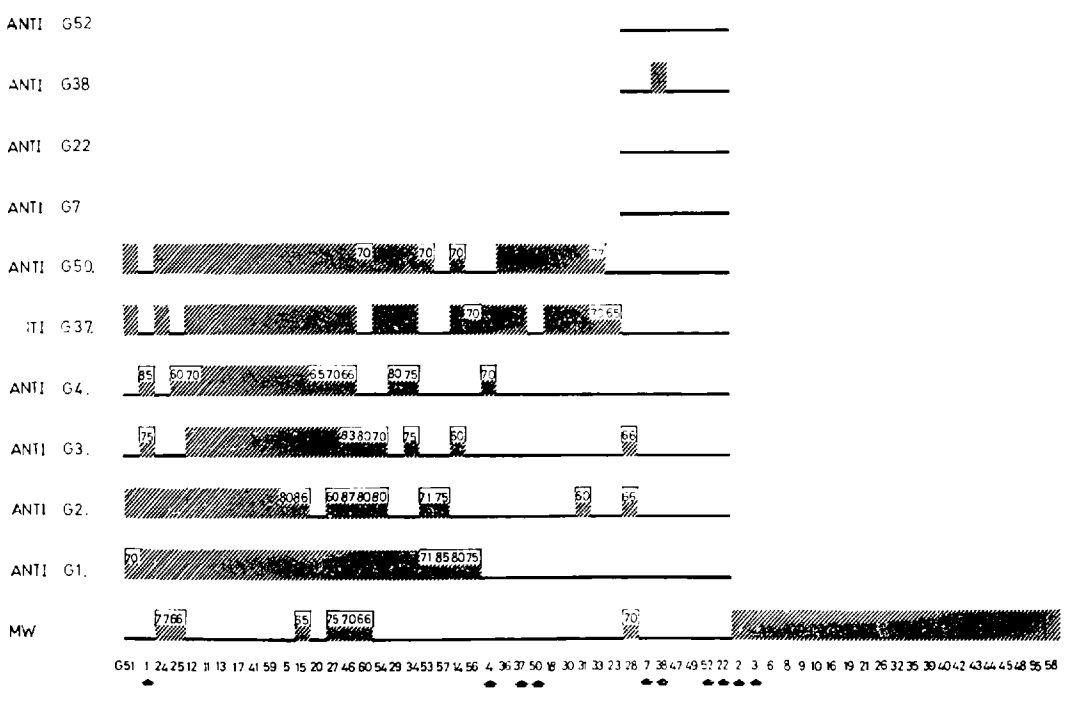


FIG. 2. *Sensitivities of 60 strains of Neisseria gonorrhoeae to 1 normal human serum (MW) and 10 immune rabbit sera. Arrows indicate strains used to prepare antisera. Solid black lines indicate no significant killing. Hatched areas indicate 90% or more killed, except where inset figures indicate between 60 and 89% killed.*

await further analysis. It should be noted that anti-G7, -G22, -G38, and -G52 have not yet been tested against strains in groups I, II, and III. Their ability to kill some of the Danish strains and their hemagglutinating activity showed that they contain specific antibodies.

Two strains, which were not killed by normal human serum, were retested after they had been subcultured two or three times a week for 6 months. They remained resistant.

The serum sensitivities of five Danish strains are shown in Table 1. Strains 2c and 42981 clearly belong in group I, whereas strain 11413 probably belongs in group II. The resistant strains 1a and 44341 would have been placed in group IV were it not for their sensitivities to anti-G1 and anti-G2. There does not appear to be any correlation between the results of the bactericidal tests and the factor formulae previously described (15).

Bactericidal antibodies in normal human serum. One-third of the strains tested were sensitive to normal human serum (serum MW) alone. Although normal human sera vary in activity (Fig. 3), antibodies reacting with some gonococcal antigens must be widely distributed. Absorption of serum MW with heat-killed suspensions of various strains of bacteria (Table 2) suggests that such antigens may be found in some Neisseria species and in *E. coli*. Surprisingly little effect was produced by the strain of *N. meningitidis* tested.

The immune rabbit serum anti-G1 was not affected by absorption with the strains tested, except for *N. gonorrhoeae* strains G1 and G2 and *N. pharyngis-sicca*.

Antigens involved in the bactericidal reaction. The heterogeneity and complexity of gonococcal antigens is shown in Table 3. Strains G1, G29, G34, and G36 were resistant to normal human serum alone but, with the exception of G36, were killed on the addition of anti-G1 serum. Absorption of anti-G1 with the sensitive strains G29 or G34 largely removed all bactericidal activity. Absorption with the resistant strain G36 made the serum ineffective against G29 and less effective against G34 but did not significantly alter the killing of G1. Absorption with the homologous strain G1 removed all bactericidal activity.

The same absorbed sera were tested for their ability to agglutinate sheep red cells coated with G1 lipopolysaccharide (Table 3). Hemagglutinating activity was absorbed not only by strains G1 and G29, as expected, but also by G36. This suggests that, although all four strains have major lipopolysaccharide antigens in common, G1 has an additional antigen involved in killing. This antigen could be another lipopolysaccharide present in such small quantities that it did not reach the red cells in the sensitization procedures, though it was adequate in situ for the bactericidal reaction. Alternatively, it could be of a different chemical nature.

Bactericidal antibodies could be removed from antiserum to G1 by absorption with live G1. The absorbing antigens resisted heat and trypsin (Table 4). Control absorptions with heated samples of strain G36 did not affect bactericidal activity. Antisera to strains G1 and G50, absorbed five times with sheep red cells coated with purified lipopolysaccharide from strains G1 and G36, respectively, showed reduced bactericidal activity (Fig. 4). Specific hemagglutinating activity was almost completely removed. Control sera absorbed with unsensitized sheep red cells were not significantly affected.

The immune sera raised against live subcultured gonococci were also tested for hemagglutinins against sheep red cells coated with lipopolysaccharides from five strains of gonococci (Table 5). Although all the cells were sensitized in the same way, they probably differed in agglutinability, whereas the antisera must also have differed in potency. However, where a serum could be tested against its homologous lipopolysaccharide, the hemagglutination titer was satisfactory.

Even allowing for variations due to these

TABLE 1. *Serum killing of Danish strains of Neisseria gonorrhoeae*

Strain no.	Factor formula	Per cent killed by[a]										
		Normal (MW)	Anti-G1	Anti-G2	Anti-G3	Anti-G4	Anti-G37	Anti-G50	Anti-G7	Anti-G22	Anti-G38	Anti-G52
1a	V	60	92	50	10	0	0	0	0	0	0	0
2c	(V), IX	>99	NT[b]	NT	NT	NT	NT	NT	NT	NT	NT	NT
42981	X, (VI), IX	>99	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
11413	III, VI	0	>99	>99	75	90	90	96	97	95	95	95
44341	IX, XII	70	>99	>99	0	0	50	50	50	83	15	0

[a] Per cent killed by antisera listed was calculated by comparison with that produced by normal human serum.

[b] Not tested.

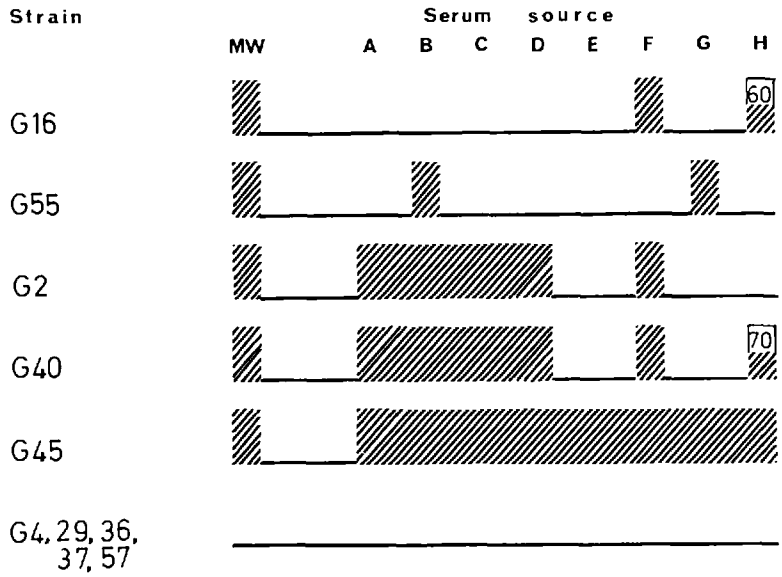


FIG. 3. *Bactericidal antibodies to Neisseria gonorrhoeae in normal human sera. Solid black lines indicate no significant killing. Hatched areas indicate 90% or more killed, except where inset figures indicate 60 or 70% killed.*

TABLE 2. *Absorption of bactericidal antibodies from normal human and immune rabbit serum by Neisseria species, Escherichia coli, and Pseudomonas aeruginosa*

Serum absorbed with	Per cent kill at 30 min of	
	Strain G2, tested with normal human serum (MW)	Strain G1, tested with rabbit anti-G1
N. gonorrhoeae G1	>90	0
N. gonorrhoeae G2	0	0
N. flavescens	>90	>99
N. meningitidis	66	>99
N. catarrhalis	33	>99
N. pharyngis-flavus	0	>99
N. pharyngis-sicca	0	0
E. coli WF96	0	NT[a]
P. aeruginosa R133	>90	>99

[a] Not tested.

TABLE 3. *Effects on bactericidal and hemagglutination titers of immune sera of absorption with heterologous strains of Neisseria gonorrhoeae*

Antibody	Absorbing strain	Percentage killed at 30 min in strains				Hemagglutination titer aganst G1 lipopolysaccharide
		G1	G29	G34	G36	
Anti-G1		99	98	98	0	640
Anti-G1	G1	0	0	0	ND[a]	<40
Anti-G1	G29	40	0	0	ND	<40
Anti-G1	G34	0	0	33	ND	ND
Anti-G1	G36	92	0	70	ND	40
Normal human serum		0	0	0	0	16

[a] Not done.

TABLE 4. *Nature of the gonococcal antigens subserving the bactericidal reaction*

Anti-G1 serum absorbed with	Killing of G1
G1, live	0
G1, 100 C, 1 hr	0
G1, 121 C, 2 hr	0
G1, 100 C, 0.5 hr, NaCl	0
G1, trypsin	0
G36, 100 C, 1 hr	>90

factors, it is clear that cross-reactions occurred and it is instructive to compare them with the results of the bactericidal tests.

Strain G1 was readily killed by anti-G1 and anti-G2, doubtfully by anti-G3 and anti-G4, and not at all by anti-G37 and anti-G50. The hemagglutination titers corresponded.

Strain G2 was killed by many normal human

sera, and its lipopolysaccharide reacted with all the immune sera. Strain G36 was killed by anti-G37 and anti-G50 but not by anti-G4. Its lipopolysaccharide, however, reacted with all three sera. Strains G22 and G38 were not killed by any antisera, although their lipopolysaccharides reacted with several. Whenever tested, therefore, antisera killing a given strain always reacted with the corresponding lipopolysaccharide, but positive hemagglutination was not always paralleled by killing.

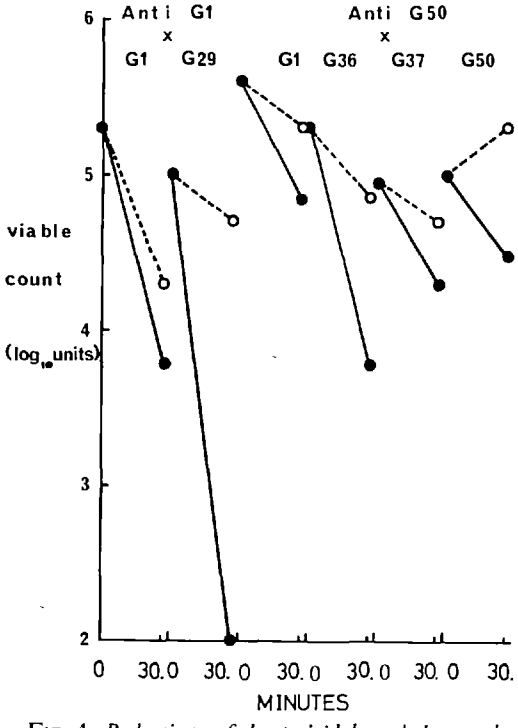


FIG. 4. *Reduction of bactericidal and hemagglutinating activity after absorption of immune sera with lipopolysaccharide-coated red cells. Viable counts with unabsorbed serum (●); viable counts with absorbed serum (○). Anti-G1 absorbed with G1; anti-G50 absorbed with G36. The hemagglutination titer of anti-G1 was 10,000 before absorption and 4 after. The corresponding titers of anti-G50 were 640 and 4.*

DISCUSSION

By means of the bactericidal reactions, 60 strains of *N. gonorrhoeae* can be separated into four main groups. These groups are not homogeneous, and it is evident that additional groups and subdivisions could be defined, given further work on the same lines. As the bactericidal reaction is tedious to perform and, particularly in relation to the gonococcus, difficult to standardize, it would be preferable to detect the antigens involved by some simpler means. In addition, antigens used as taxonomic criteria ought to be clearly defined chemically.

The reactions with normal sera show that cross-reacting antigens occur in other species of *Neisseria* and even in other genera. Moreover, several different antigens are involved since not all normal sera behaved similarly. From the absorption tests, it seems likely that the immune sera were more species-specific.

We have given reasons for believing that the main antigens concerned in serum killing are lipopolysaccharides. However, tests with red cells coated with lipopolysaccharides showed that both normal and immune sera reacted with a far wider range of organisms in the hemagglutination than in the bactericidal reaction. Since the latter is generally taken as the more sensitive test, the most likely explanation is that lipopolysaccharides may be present in gonococci but not necessarily accessible to antibody. This could be due to purely quantitative variations or to the presence of blocking antigens in the intact organisms. The absorption experiments would fit in with this hypothesis, as well as suggesting that there may be several antigenically different lipopolysaccharides per strain. It is also possible, though perhaps less likely, that in some strains the bactericidal reaction is based on other types of

TABLE 5. *Hemagglutination titers of immune antigonococcal sera tested against sheep red cells sensitized with gonococcal lipopolysaccharides*

Sensitizing lipopolysaccharide from strain	Hemagglutinin titers of antisera to strain										Pooled normal human serum
	G1	G2	G3	G4	G7	G22	G37	G38	G50	G52	
G1	1,280	640	40	40	320	80	40	<40	40	640	16
G2	2,560	1,280	1,280	1,280	5,120	2,560	2,560	2,560	640	5,120	16
G22	1,280	<40	640	80	1,280	2,560	80	2,560	1,280	1,280	16
G36	40	40	40	320	80	<40	320	<40	80	<40	8
G38	320	<40	640	160	640	320	160	640	80	640	32

antigen. For example, it would be difficult to explain the sensitivities of strain G36 simply on the presence of blocking antigen. If this blocked anti-G4 which can react with G36 lipopolysaccharide, it ought to have blocked anti-G37 and anti-G50 as well.

If the outcome of bactericidal reactions to a given set of antisera is the result of several categories of antigens, it is unlikely that any other single test would give the same pattern. Further analysis is needed, but, from the diagnostic point of view, it is clear that any serological test for gonorrhoea must not be based on only one or two strains of gonococci but must take into account their antigenic heterogeneity.

It is tempting to compare the antigenic makeup of gonococci with the O and K or Vi systems of enterobacteria. How far such antigens are responsible for virulence is still debated in the enterobacteria (10) and is pure speculation in *N. gonorrhoeae*.

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LITERATURE CITED

1. Amies, C. R., and M. Garabedian. 1967. An easily prepared selective medium for the cultivation of *Neisseria gonorrhoeae*. Brit. J. Vener. Dis. 43:137–139.
2. Chacko, C. W. and G. M. Nair. 1969. Serodiagnosis of gonorrhoea with a microprecipitin test using a lipopolysaccharide antigen from *N. gonorrhoeae*. Brit. J. Vener. Dis. 45:33–39.
3. Chanarin, I. 1954. An investigation of *Neisseria gonorrhoeae* by a red cell sensitization technique. J. Hyg. 52:426–443.
4. Cohen, I. R. 1967. Natural and immune human antibodies reactive with antigens of virulent *Neisseria gonorrhoeae*: immunoglobulins G, M, and A. J. Bacteriol. 94:141–148.
5. Danielsson, D. 1965. The demonstration of *N. gonorrhoeae* with the aid of fluorescent antibodies 4. Studies by immunofluorescence and double diffusion-in-gel technique on the antigenic relationship between *N. gonorrhoeae* and other Neisseria strains. Acta Pathol. Microbiol. Scand. 64: 267–276.
6. Danielsson, D. G., J. D. Schmale, W. L. Peacock, Jr., and J. D. Thayer. 1969. Antigens of *Neisseria gonorrhoeae*: characterization by gel filtration, complement fixation, and agar-gel diffusion of antigens of gonococcal protoplasm. J. Bacteriol. 97:1012–1017.
7. Davies, D. A. L., M. J. Crumpton, I. A. MacPherson, and A. M. Hutchison. 1958. The adsorption of bacterial polysaccharides by erythrocytes. Immunology 1:157–171.
8. Deacon, W. E., W. L. Peacock Jr., E. M. Freeman, and A. Harris. 1959. Identification of *Neisseria gonorrhoeae* by means of fluorescent antibodies. Proc. Soc. Exp. Biol. Med. 101:322–325.
9. Gerhardt, P., and C.-G. Hedén. 1960. Concentrated culture of gonococci in clear liquid medium. Proc. Soc. Exp. Biol. Med. 105:49–51.
10. Glynn, A. A., and C. J. Howard. 1970. The sensitivity to complement of strains of *Escherichia coli* related to their K antigens. Immunology 18:331–346.
11. Kabat, E. A., and M. M. Mayer. 1961. Experimental immunochemistry, p. 135, 2nd ed. Charles C Thomas, Publisher, Springfield, Ill.
12. Maeland, J. A. 1968. Antigenic properties of various preparations of *Neisseria gonorrhoeae* endotoxin. Acta Pathol. Microbiol. Scand. 73:413–422.
13. Maeland, J. A. 1969. Immunochemical characterization of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae*. Acta Pathol. Microbiol. Scand. 76:484–492.
14. Martin, J. E., Jr., W. L. Peacock, Jr., G. Reising, D. S. Kellogg, Jr., E. Ribi, and J. D. Thayer. 1969. Preparation of cell walls and protoplasm of *Neisseria* with the Ribi cell fractionator. J. Bacteriol. 97:1009–1011.
15. Reyn, A. 1949. Serological studies on gonococci. II Cross-absorption experiments and factor serum determinations. Acta Pathol. Microbiol. Scand. 26:234–251.
16. Schaedler, R. W., R. Dubos, and R. Costillo. 1965. The development of the bacterial flora in the gastrointestinal tract of mice. J. Exp. Med. 122:59–66.
17. Schmale, J. D., D. G. Danielsson, J. F. Smith, L. Lee, and W. L. Peacock, Jr. 1969. Isolation of an antigen of *Neisseria gonorrhoeae* involved in the human response to gonococcal infection. J. Bacteriol. 99:469–471.
18. Tauber, H. and H. Russell. 1961. Biochemistry of *N. gonorrhoeae* endotoxin. Bull. World Health Organ. 24:385–386.
19. Wilson, G. S., and A. A. Miles. 1964. Principles of bacteriology and immunity, p. 678. vol. 1, 5th ed. Edward Arnold, London.

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Gonococci in Urethral Exudates possess a Virulence Factor lost on Subculture

MAN is uniquely susceptible to gonorrhoea, but antibodies present in normal human sera are bactericidal for cultured gonococci[1-3]. The ineffectiveness of these antibodies in the natural infection may be explained if gonococci growing in the host possess a factor, perhaps a surface antigen, which protects the organism from the bactericidal action of serum. There is some evidence that *Neisseria gonorrhoeae* loses virulence factors during growth *in vitro* because enormous doses of cultured gonococci were required to infect volunteers[4,5] while organisms recovered from the resulting urethral discharges exhibited enhanced virulence[6]. Another, less likely, suggestion is that gonococci only infect individuals lacking bactericidal antibodies to the strain concerned. To distinguish between these possibilities we have tested the ability of serum from men with gonorrhoea of less than 3 days duration to kill the gonococci present in the urethral exudate and the same organism after subculture.

Exudates were collected into mineral salt solution (Dulbecco) and homogenized using a 'Teflon' grinder[7] to rupture any cells present. The gonococci were washed twice, resuspended in 0·5 ml. of Dulbecco's solution, 0·1 ml. of this suspension containing some 10^3 to 10^5 gonococci added to 0·4 ml. of the corresponding patient's serum and the number of surviving gonococci counted after incubation for 1 h at 37° C. The serum was stored at −70° C and the experiment repeated using the same gonococcus after subculture. The techniques used in the preservation, culture and viable counting of gonococci have been described previously[3]. Viable counts are expressed as logarithms to base 10.

In twenty such experiments the logarithm of the average number of gonococci in exudates added to the serum was 4·30 and the average number recovered after incubation for 1 h was again 4·30 (Fig. 1). Minor differences in individual experiments can be explained by the tendency of gonococci in exudates to form clumps. When log phase subcultures were incubated in the corresponding patient serum, seventeen out of the twenty gonococcal isolates showed a greater than 90 per cent kill (Fig. 1). Thus most patients with acute gonorrhoea possess bactericidal antibodies active against the infecting strain grown *in vitro*, but the same antibodies fail to kill the gonococci present in urethral pus. These antibodies were not induced by the infection because identical

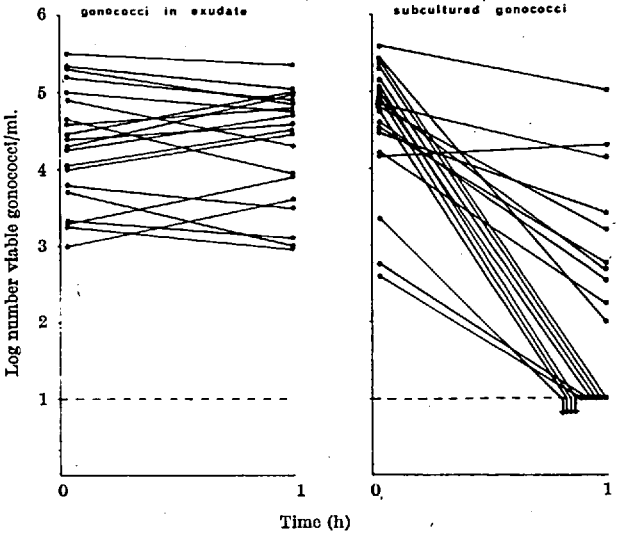


Fig. 1. The effect of patients' serum on the corresponding strains of *N. gonorrhoeae* both in the native exudates and after subculture. Here (and also in Figs. 2 and 3) each line represents a separate experiment so that the group of lines in each graph gives an indication of the scatter of the results.

results were obtained using the serum of a normal individual (M. E. W.).

We have used a streptomycin resistant gonococcus (G11) to exclude the possibility that the washed exudates were anticomplementary or that substances present in them protected the organisms from the bactericidal action of serum. About 10^6 organisms of strain G11 were mixed with 0·1 ml. of the same washed exudates and added to 0·4 ml. of normal human serum (M. E. W.) containing a 1 : 100 dilution of each of ten rabbit antisera. Viable counts were made on media containing 25 μg/ml. streptomycin. The results (Fig. 2) show that in the presence of exudate, strain G11 was rapidly destroyed by antibody plus complement, the average fall in viable count exceeding 5 log units after 1 h incubation.

Gonococci recovered from exudates proved to be resistant to the bactericidal action of complement plus polyvalent rabbit antisera; the log average fall in viable count, 0·69, was less than the drop in count arising from agglutination when subcultured gonococci were exposed to antiserum alone (Fig. 3). Apart from one strain, G87, the same gonococcal isolates after subculture were rapidly killed by the antiserum plus complement. A few gonococcal isolates have proved resistant to rabbit antiserum raised against the corresponding strain, although the

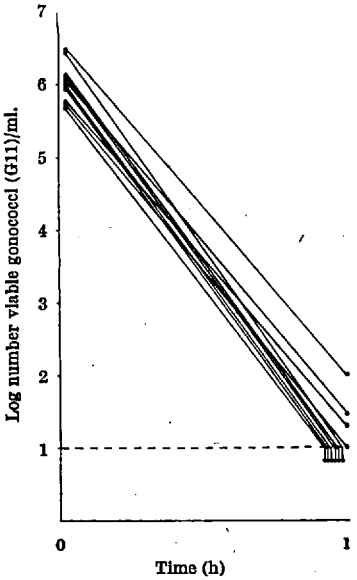


Fig. 2. The effect of rabbit antiserum plus complement on a streptomycin resistant strain of *N. gonorrhoeae* (G11) in the presence of urethral exudate. Viable counts were made on media containing 25 μg/ml. streptomycin.

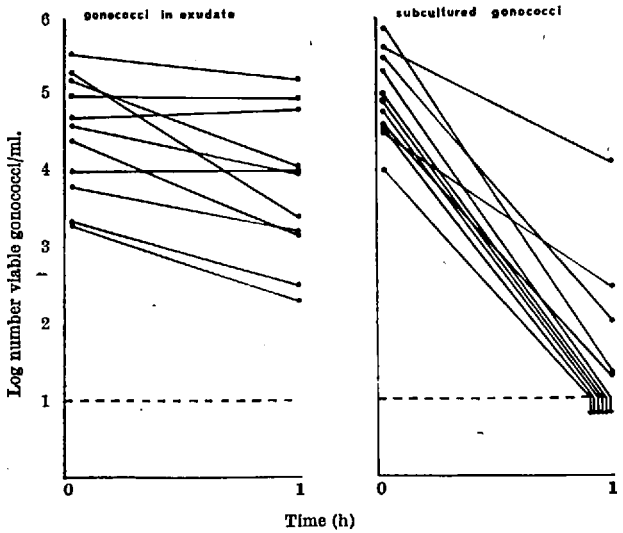


Fig. 3. The effect of polyvalent rabbit antisera with human serum as a source of complement on strains of *N. gonorrhoeae* both in the native exudate and after subculture.

antiserum was bactericidal for other gonococci. This finding is analogous to the K antigens found in the *Enterobacteriaceae* where some organisms possessing large amounts of K antigen resist the bactericidal action of specific "anti-K" antibodies[8] (unpublished results of Howard and A. A. G.). Any protective factor present on gonococci growing within the host should not be confused with the "K-antigen" described by Deacon *et al.*[9] which was induced by growth *in vitro* and seems to be unrelated to the virulence of gonococci[10].

Thus we have shown that gonococci growing in the host possess a protective factor which enables them to resist killing by natural antibodies and complement. This factor, which may be a surface antigen comparable with the K antigens of coliforms, was quickly lost on subculture. The fact that occasional strains of gonococci retain their resistance to serum killing after subculture might, however, form the basis of attempts to isolate this protective factor and investigate its immunogenic properties.

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[1] Spink, W. W., and Keefer, C. S., *J. Clin. Invest.*, **16**, 169 (1937).
[2] Gordon, J., and Johnstone, K. I., *J. Path. Bact.*, **50**, 483 (1940).
[3] Glynn, A. A., and Ward, M. E., *WHO/VDT/Res/Gon*, **69**, 34 (1969).
[4] Hill, J. H., *Amer. J. Syph. Gonorrhoea Vener. Dis.*, **27**, 733 (1942).
[5] Kellogg, D. S., and Thayer, J. D., *Ann. Rev. Med.*, **20**, 323 (1969).
[6] Mahoney, J. F., Van Slyke, C. J., Cutler, J. C., and Blum, H. L., *Amer. J. Syph. Gonorrhoea Vener. Dis.*, **30**, 1 (1946).
[7] Pierce, C. H., Dubos, R. J., and Shaeffer, W. B., *J. Exp. Med.*, **97**, 189 (1953).
[8] Nagington, J., *Brit. J. Exp. Path.*, **37**, 385 (1956).
[9] Deacon, W. E., Peacock, W. L., Freeman, E. M., and Harris, A., *Proc. Soc. Exp. Biol. Med.*, **101**, 322 (1959).
[10] Kellogg, D. S., Peacock, W. L., Deacon, W. E., Brown, L., and Pirkle, C. I., *J. Bact.*, **85**, 1274 (1963).

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