Gonococci Causing Disseminated Gonococcal Infection Are Resistant to the Bactericidal Action of Normal Human Sera

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ABSTRACT The susceptibility of strains of *Neisseria gonorrhoeae* to the bactericidal action of normal human sera was determined for isolates from patients with disseminated gonococcal infection and uncomplicated gonorrhea. Serum susceptibility was correlated with penicillin susceptibility and auxotype. 38 of 39 strains (97%) of *N. gonorrhoeae* from Seattle patients with disseminated gonococcal infection were resistant to the complement-dependent bactericidal action of normal human sera. 36 of these were inhibited by ≤0.030 μg/ml of penicillin G and required arginine, hypoxanthine, and uracil for growth on chemically defined medium ($Arg^-Hyx^-Ura^-$ auxotype). 12 of 43 isolates from patients with uncomplicated gonorrhea were also of the $Arg^-Hyx^-Ura^-$ auxotype, inhibited by ≤0.030 μg/ml of penicillin G, and serum resistant. Of the 31 remaining strains of other auxotypes isolated from patients with uncomplicated gonorrhea, 18 (58.1%) were sensitive to normal human sera in titers ranging from 2 to 2,048. The bactericidal action of normal human sera may prevent the dissemination of serum-sensitive gonococci. However, since only a small proportion of individuals infected by serum-resistant strains develop disseminated gonococcal infection, serum resistance appears to be a necessary but not a sufficient virulence factor for dissemination. Host factors such as menstruation and pharyngeal gonococcal infection may favor the dissemination of serum-resistant strains. Since serum-resistant $Arg^-Hyx^-Ura^-$ strains are far more frequently isolated from patients with disseminated gonococcal infection than serum-resistant strains of other auxotypes, $Arg^-Hyx^-Ura^-$ strains may possess other virulence factors in addition to serum resistance.

INTRODUCTION

Disseminated gonococcal infection (DGI),[1] usually associated with arthritis and dermatitis occurs in an estimated 1–3% of patients with gonorrhea in Seattle, Wash. (1). Most strains recovered from patients with disseminated disease in Seattle are susceptible to ≤0.030 μg/ml of penicillin G (2) and require arginine, hypoxanthine, and uracil for growth (3). In contrast, strains that produce localized gonococcal infection are usually more resistant to penicillin and are of varying auxotypes (3). These differences suggest that strains which cause DGI are unique and might possess virulence factor(s) that mediate dissemination.

Virulence and resistance to the complement-dependent bactericidal activity of normal human serum appear to be closely associated for many gram-negative bacteria (4–9). Glynn and Ward showed that some strains of gonococci were resistant to the bactericidal action of normal human sera (10), but did not relate this property to virulence. Spink and Keefer reported in 1937 that fresh defibrinated blood from healthy men killed gonococci isolated from uncomplicated genital infections more efficiently than systemic gonococcal isolates (11). The present study extends the observations of Spink and Keefer and examines the

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[1] *Abbreviations used in this paper*: $Arg^-Hyx^-Ura^-$ auxotype, arginine, hypoxanthine, and uracil; CFU, colony-forming unit; CH_{50}, reciprocal of the serum dilution at which 50% of the indicator erythrocytes are hemolyzed; DGI, disseminated gonococcal infection.

TABLE I

Strains of N. Gonorrhoeae Studied for Susceptibility to the Bactericidal Action of Human Sera

Syndrome	Auxotype*	Total no. of isolates	Pen. MIC‡		Isolation site§		Serum source‖		
			≤0.030	>0.030	Systemic	Local	Individual	Pool	Acute/Conv
DGI	$Arg^-Hyx^-Ura^-$	36	36	0	12	24	7	32	6
	Proto	2	0	2	1	1	1	1	0
	Pro^-	1	0	1	1	0	0	1	0
Uncomplicated gonorrhea (includes strains 9 and F62)	$Arg^-Hyx^-Ura^-$	12	12	0	0	12	0	12	0
	Proto	17	2	15	0	17	3	15	1
	Pro^-	11	1	10	0	11	1	10	0
	Arg^-	2	0	2	0	2	0	2	0
	Pro^-Arg^-	1	0	1	0	1	1	1	1

* Auxotype: $Arg^-Hyx^-Ura^-$, requires arginine, hypoxanthine, and uracil for growth; Proto, prototrophic for proline, arginine, hypoxanthine, uracil, and methionine; Pro^-, proline requiring; Arg^-, arginine requiring.

‡ Pen. MIC, Minimum inhibitory concentration of penicillin G, expressed in micrograms per milliliter.

§ Isolation site: Systemic, blood, joint effusion, or skin pustule; Local, urethra, cervix, or anal canal.

‖ Serum source: Individual, strain tested individually with 20 normal human sera for serum susceptibility; Pool, strain tested with pooled normal human sera for serum susceptibility; Acute/Conv, strain tested with acute and convalescent sera from six patients with DGI. Numbers exceed total number of strains because some strains were tested by more than one serum source.

relationship of auxotype and penicillin sensitivity to the resistance of gonococci to the bactericidal action of normal human sera.

METHODS

Bacteriological techniques

Selection, identification, and storage of organisms tested. Strains of *Neisseria gonorrhoeae* used in this study included strains F62 and 9, supplied by Dr. Douglas Kellogg, Center for Disease Control, Atlanta, Ga., 39 isolates from Seattle patients with DGI, and 41 isolates from Seattle patients with uncomplicated gonorrhea (Table I). The 39 isolates from patients with DGI included 14 recovered from blood, synovial effusion, or a skin pustule, and 25 recovered from the urethra or cervix of patients with typical manifestations of the gonococcal arthritis-dermatitis syndrome (1). The 41 strains of gonococci from patients with uncomplicated gonorrhea were isolated on Thayer-Martin medium (12) from urethra, endocervix, or anal canal. All Seattle strains were isolated during 1971–1975.

Isolates were identified as *N. gonorrhoeae* by oxidase reaction, Gram stain, and sugar fermentation reactions (13) and preserved by lyophilization (14) or freezing at −70°C in a medium consisting of 5% (wt/vol) bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) and 5% (wt/vol) monosodium glutamate (15). All clinical isolates were tested in the bactericidal assay after the fewest in vitro passes required for isolation in pure culture and confirmation of species and were maintained as colony variants T1 or T2 (16, 17), except for strain 1947 which was tested as colony variant T3. In addition, eight of the genital isolates were tested after 1 and 10 in vitro passes to assess the effect of subculturing on their susceptibility to the bactericidal action of serum. Strains F62 and 9 were laboratory strains which had been passed multiple times in vitro before bactericidal testing.

Auxotyping and penicillin susceptibility testing. Gonococci were auxotyped by the method of Catlin (18) and Carifo and Catlin (19) as modified by Knapp and Holmes (3), and their susceptibility to penicillin G (Table I) was determined as the minimum inhibitory concentration (MIC) by an agar dilution technique, using serial $\log_2$ dilutions of penicillin G in GC base medium (Difco Laboratories, Detroit, Mich.) with the addition of 1% defined supplement (20). The suspension of organisms was adjusted to an optical density of 0.15 (560 nm, 1 cm lightpath) in a Spectronic 20 Colorimeter (Bausch & Lomb, Inc., Scientific Optical Products Div., Rochester, N. Y.) and was inoculated using a Steers replicator (21).

10 strains were assessed at the time of isolation and after 6 and 12 mo of storage for stability of auxotype and penicillin MIC. Their nutritional profiles were unchanged during the period of study and their susceptibility to penicillin varied within a single $\log_2$ dilution. No change in the requirement of 13 strains of arginine, hypoxanthine, and uracil was observed after 4–10 in vitro passages.

Preparation of bacterial suspensions for use in the bactericidal assay. All comparisons between strains of their susceptibility to the bactericidal action of normal human sera were made with organisms grown in identical media with bacterial suspensions prepared in a standardized manner. Specifically, all strains of each auxotype were harvested with a sterile swab from 18-h cultures on GC agar medium containing 1% defined supplement, suspended in Tryptic Soy Broth (Difco Laboratories) to an optical density of 0.10 at 560 nm, and 100 μl was inoculated onto GC base medium containing defined supplement. Plates were incubated at 36.5°C in 3% CO_2 and were harvested by washing the plates with Tryptic Soy Broth after 5 h of incubation. These strains were determined, by serial replicate plate counts of viable organisms, to be in early log phase growth 5 h after inoculation of agar.

Individual strains tested for change in serum susceptibility after 1 and 10 in vitro passes (strains 1 through 8 of Table III) and with acute and convalescent sera (strains F62 and 6366 of Table VI) were harvested from 18-h subcultures, inoculated into Tryptic Soy Broth, and grown at 36.5°C in air in a nepholometer flask (Bellco Glass, Inc., Vineland, N. J.) on a model G25 gyrotory shaker incubator (New

Brunswick Scientific Co., Inc., New Brunswick, N. J.). Growth phase was monitored by change in optical density at 560 nm in a Coleman Junior Spectrophotometer (Coleman Instruments Div., Perkin Elmer Corp., Oak Brook, Ill.), and organisms were harvested 1 h after entering log phase growth. Organisms harvested from broth or plates were suspended in Tryptic Soy Broth, agitated with a Vortex Genie Mixer (Scientific Industries, Inc., Bohemia, N. Y.), and appropriate dilutions for the bactericidal assay were made in Medium 199 containing glutamine and sodium bicarbonate, pH 7.4 (Grand Island Biological Co., Grand Island, N. Y.).

Sera

Blood from 10 men and 10 women with no history of gonorrhea or of laboratory exposure to *N. gonorrhoeae* (Table I) was allowed to clot for 60 min at 37°C and sedimented by centrifugation (1,500 *g* for 20 min at 4°C) in a model PR-2 centrifuge (International Equipment Co., Needham Heights, Mass.), and the serum was filter sterilized with a Millex 0.45-μm filter unit (Millipore Corp., Bedford, Mass.) and frozen in 2-ml aliquots at −20° and −70°C. Serum was heat inactivated at 56°C for 30 min before use in the bactericidal assay.

Sera from six patients with DGI (Table I) were obtained immediately before the initiation of antibiotic therapy and again at varying intervals after antibiotics had been discontinued. In addition, acute sera obtained from 18 patients presenting with gonococcal arthritis were tested for complement activity.

Complement

Fresh sera from many species, including newborn guinea pigs, adolescent rabbits, and adult humans, proved unsatisfactory as sources of complement, because all such sera were bactericidal for many strains of *N. gonorrhoeae*. The source of complement for the bactericidal test was a 25-yr-old Caucasian man with X-linked agammaglobulinemia (23–24) whose serum lacked bactericidal activity for all strains of *N. gonorrhoeae* tested but supported the bactericidal action of heat-inactivated normal human sera for serum-sensitive strains. Serum obtained just before the donor's monthly gamma globulin infusion was stored at −70°C. The concentration of immunoglobulins in this serum, expressed in milligrams per 100 milliliters, was determined by quantitative radial immunodiffusion (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.) to be: IgG < 20, IgM < 5, and IgA < 5. C3 and C4 levels were normal by quantitative radial immunodiffusion (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif. and Meloy Laboratories, Inc., Springfield, Va., respectively), and the total hemolytic complement activity, expressed in CH_{50} units (reciprocal of serum dilution at which 50% of the indicator erythrocytes are hemolyzed), was determined by a modification of the method of Mayer (25). Only samples having 100 or more CH_{50} units per milliliter were used.

Serum bactericidal assay

The serum bactericidal test was performed in a microtiter system using disposable U-well trays and 50-μl diluters (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.) and Eppendorf pipettes (Brinkmann Instruments, Inc., Westbury, N. Y.). Plastic materials were sterilized by exposure to ethylene oxide (H. W. Andersen Products, Inc., Oyster Bay, N. Y.) for 16 h. The reaction mixture had a total volume of 150 μl and consisted of one part diluted serum, one part bacterial suspension (approximately 600 colony-forming units [CFUs] in 50 μl Medium 199), and one part complement diluted 1:2 or 1:3 in Medium 199, to give a final complement activity >20 CH_{50} U/ml of reaction mixture. The sera were doubly diluted from 1:2 through 1:16,384, and each dilution was performed in triplicate for each strain tested. Complement controls (without added serum) and heat-inactivated serum controls (without added complement) were included in each experiment. The trays were sealed with sterile transparent tape (Cooke Laboratory Products Div.), shaken horizontally to mix the reactants, and incubated at 36.5°C on a gyratory shaker for 60 min. Sterile Pasteur pipettes were then used to deliver a drop of fluid from each well onto dried, warmed GC base medium with added supplement. The plates were incubated for 18 h at 36.5°C in the presence of 3% CO_2 and moisture. The pipettes delivered approximately 50 μl/drop, and in the absence of bactericidal activity such a drop contained approximately 200 CFUs.

Agglutination ("clumping") of gonococcal colony variants T1 and T2 in liquid media was diminished by vortex agitation of the bacterial suspension and use of a low concentration of organisms in the bactericidal assay. Both an electronic particle counter study (Model ZH Coulter Counter, Coulter Electronics Inc., Hialeah, Fla.) and observation by dark-field microscopy determined that the frequency distribution of bacterial aggregates (and organisms per aggregate) was equal for the serum and complement controls, the serum titers at which greater than a 75% reduction in CFUs occurred (significant killing), and serum titers at which no reduction in CFUs was noted.

Kinetic killing experiments

The rate of the complement-dependent bactericidal action of normal human sera was studied for four strains from patients with disseminated infection and four from patients with uncomplicated infection. Undiluted pooled serum stored at −70°C was added in 50-μl amounts to each microtiter well, and approximately 1,000 CFUs in log phase growth were added in 5-μl volume to each well. At predetermined time intervals over a 90-min period, 200 μl of Medium 199 was added, and the well contents were cultured immediately as described above. Heat-inactivated serum and buffer controls were cultured concurrently.

Interpretation of bactericidal test

Significant bactericidal activity was defined as a 75% reduction in CFUs when compared with the heat-inactivated serum control. The bactericidal titer was defined as the reciprocal of the greatest dilution of serum producing significant killing. In kinetic killing experiments, the rate of the bactericidal reaction was characterized by the percentage of surviving bacteria at each sampling time interval when compared to the concurrently sampled heat-inactivated serum controls.

RESULTS

Bactericidal titer of normal sera for gonococci recovered from patients with urethral, cervical, or disseminated infection. Normal human sera from 20

healthy men and women without a history of gonorrhea were bactericidal (serum titer ≥ 2) for each of five strains of gonococci isolated from patients with uncomplicated urethral or cervical infections. These included two laboratory-adapted strains (F62 and 9) and three recent isolates (Table II). None of these strains required arginine, hypoxanthine, or uracil for growth. As shown in Table II, these five strains showed marked strain-related differences in their susceptibility to the bactericidal action of normal human sera, but there was relatively less variation in the activity of the 20 sera against any individual strain. The bactericidal activity of male and female sera for these strains was similar, with geometric mean titers of 44.63 (female) and 45.25 (male). No ABO blood group-associated differences in bactericidal activity were found.

Eight strains of gonococci isolated from patients with the DGI syndrome were resistant (serum titer < 2) to each of the 20 sera tested (Table II). Seven of these strains required arginine, hypoxanthine, and uracil for growth and one was prototrophic.

The susceptibility of eight anogenital isolates to the complement-dependent bactericidal activity of pooled normal human sera was determined after 1 and 10 selective passages in vitro as T1 or T2 colonies on synthetic medium (Table III). A fourfold change in titer occurred in only one instance (strain 6).

Kinetics of the bactericidal action of pooled normal human sera on isolates from urethral, cervical, and disseminated infections. The bactericidal reaction in pooled sera that had been stored at −70°C to preserve complement was characterized for strains isolated from disseminated infections by a variable lag period (22–60 min) during which the CFU count did not change, followed by the onset of killing (Fig. 1). Strain F62, an urethral isolate, was killed within 2 min without a detectable initial delay. Strain 2017, isolated from blood, and strain 1947, isolated from a joint effusion were only partially killed (30 and 4%, respectively) at the end of the 90-min reaction (Fig. 1).

All isolates from patients with disseminated infections tested in the kinetic study had been shown to be serum resistant (bactericidal titer < 2) in the stand-

TABLE II

Bactericidal Titer of 20 Normal Human Sera for Five Strains of N. Gonorrhoeae Isolated from Uncomplicated Infections and Eight Strains Isolated from DGI*

Serum source			Strains isolated from uncomplicated infections					Strains isolated from disseminated infections	
Sex	ABO Blood type	Auxotype:‡	Pro⁻Arg⁻ F62	Pro⁻ 5060	Proto 9	Proto 6366	Proto 6367	Arg⁻Hyx⁻Ura⁻ 7 strains§	Proto 1947
F	O		1024	64	8	8	2048	<2	<2
F	A		2048	4	8	8	512	<2	<2
F	A		256	4	4	8	128	<2	<2
F	O		2048	2	64	8	256	<2	<2
F	O		512	8	16	8	128	<2	<2
F	A		1024	2	4	2	128	<2	<2
F	A		1024	32	128	32	256	<2	<2
F	O		256	4	2	16	128	<2	<2
F	O		512	32	4	8	256	<2	<2
F	A		2048	4	16	16	1024	<2	<2
M	A		2048	32	32	32	1024	<2	<2
M	O		1024	2	32	8	512	<2	<2
M	A		2048	2	4	8	1024	<2	<2
M	O		256	32	4	4	128	<2	<2
M	B		1024	256	16	16	256	<2	<2
M	O		128	4	16	32	128	<2	<2
M	A		1024	4	2	2	128	<2	<2
M	O		2048	4	4	2	512	<2	<2
M	O		512	64	2	128	128	<2	<2
M	A		256	8	8	4	512	<2	<2
Geometric mean (Strain)			776.05	9.19	8.88	9.51	304.44	<2	<2

* Titer: Reciprocal of serum dilution resulting in ≥75% reduction in CFU compared to heat-inactivated serum control.

‡ Auxotype: Arg⁻Hyx⁻Ura⁻, requires arginine, hypoxanthine, and uracil for growth; Proto, prototrophic for proline, arginine, hypoxanthine, uracil, and methionine; Pro⁻, proline requiring; Arg⁻, arginine requiring.

§ All seven Arg⁻Hyx⁻Ura⁻ isolates (2017, 1385, 1384, 1896, 2025, 1939, and 6041) from patients with DGI were resistant to a 1:2 dilution of each of the 20 normal sera.

TABLE III
Serial In Vitro Passage: Effect on Bactericidal Titer

Strain	Bactericidal titer*	
	Passed X1	Passed‡ X10
1	1024	512
2	128	64
3	128	256
4	256	256
5	<2	2
6	2	8
7	<2	<2
8	2	2

* Bactericidal titer: Reciprocal of pooled normal human sera dilution resulting in ≥75% reduction in CFU compared to heat-inactivated serum control.
‡ All strains selectively passed as T1 or T2 colony types.

ard bactericidal assay (Table II) which was conducted for a 60-min reaction period and in which the lowest testable titer was one part serum diluted by two parts bacterial suspension and complement added to achieve a final activity of >20 CH_{50} U/ml. Two of four of these serum-resistant strains (1939 and 1349) were observed to be killed in the kinetic studies (99% reduction in CFU) by the end of a 70-min reaction period (Fig. 1) in the reaction mixture containing 1 part bacterial suspension to 10 parts serum (containing both antibody and complement) to more closely approximate in vivo conditions.

The onset of killing occurred later for isolates from disseminated infections than for those from uncomplicated genital infections, but differences in the rate of killing thereafter were less marked. An association was observed between bactericidal titers and killing kinetics. Those strains killed by the highest titers of normal sera had the earliest onset of killing in kinetic experiments.

Relationship of auxotype, penicillin and serum susceptibility, and syndrome. 36 of 39 strains isolated from cases of DGI in Seattle required arginine, hypoxanthine, and uracil for growth ($Arg^-Hyx^-Ura^-$ auxotype) and were susceptible to ≤0.030 μg/ml of penicillin G (Table I). All of these 36 $Arg^-Hyx^-Ura^-$ strains (100%) were resistant to the bactericidal action of pooled or individual normal human sera (Table IV). 3 of 39 isolates from Seattle patients with DGI were not of the $Arg^-Hyx^-Ura^-$ auxotype and were resistant to 0.030 μg/ml of penicillin G (Table I). All three were tested in the bactericidal assay (Table IV). Two of these were serum resistant, but one was killed at a titer of 2,048. This was a proline requiring cervical isolate from a patient with chronic arthritis, positive latex fixation test for rheumatoid factor (titer = 1:160), and hyperglobulinemia (serum globulin = 5.5 g/100 ml). Therefore, serum-resistant isolates from Seattle patients with DGI were present in 38 of 39 (97%) cases of disseminated disease.

43 cervical or urethral isolates from patients with uncomplicated gonorrhea were also tested in the bactericidal assay against individual or pooled normal human sera (Tables I and V). 12 of these were $Arg^-Hyx^-Ura^-$ strains, susceptible to ≤0.030 μg/ml of penicillin G, and all were serum resistant (bactericidal titer < 2). The remaining 31 isolates did not require arginine, hypoxanthine, and uracil for growth, and 13 of these 31 strains (41.9%) were serum resistant ($\chi^2 = 9.22$, $P < 0.005$). Exclusive of $Arg^-Hyx^-Ura^-$ strains, serum resistance, penicillin MIC, and auxotype were not uniquely associated. In contrast, all $Arg^-Hyx^-Ura^-$ strains tested (Tables IV and V), whether from patients with uncomplicated gonorrhea (12 isolates) or disseminated infection (36 isolates), were susceptible to ≤0.030 μg/ml of penicillin G and resistant to the bactericidal action of normal human sera (titer < 2).

Bactericidal activity of acute and convalescent sera from patients with DGI. The bactericidal activity of acute and convalescent sera from six patients with DGI (Table VI) was determined for the homologous strain and for two urethral isolates (strains F62 and 6366). Acute and convalescent sera from patients with disseminated infections were bactericidal for the urethral isolates in titers similar to those obtained for pooled normal human sera (Table VI). In four instances (1896, 1939, 1950, and 883) neither the acute

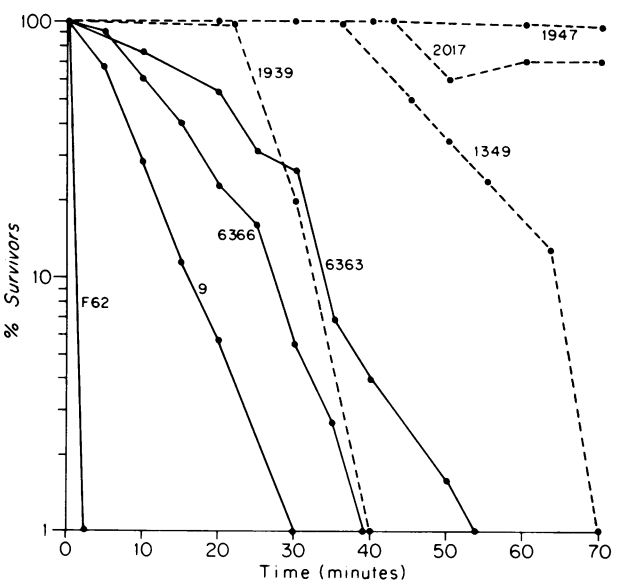

FIGURE 1 Kinetics of the bactericidal reaction. The reduction in viable bacteria by pooled normal human sera expressed as the logarithm of the percent of surviving organisms for four strains isolated from patients with uncomplicated gonorrhea (· —— ·) and four strains from patients with DGI(· - - - ·).

TABLE IV

Relationship of Auxotype, Penicillin Susceptibility, and Serum Susceptibility, of Strains Causing Disseminated Gonococcal Infection

Penicillin G MIC§	Auxotype‡	Number of strains with indicated penicillin MIC, auxotype, and serum susceptibility*					
		Arg⁻Hyx⁻Ura⁻		Proto		Pro⁻	
		Serum resistant	Serum sensitive	Serum resistant	Serum sensitive	Serum resistant	Serum sensitive
2.0				1			
1.0				1			
0.50							
0.25							
0.125							1
0.060							
0.030							
0.015		8					
0.008		19					
0.004		9					
Total		36		2			1

* Serum susceptibility: Serum resistant, serum bactericidal titer <2; Serum sensitive, serum bactericidal titer = 2,048.
‡ Auxotype: Arg⁻Hyx⁻Ura⁻, requires arginine, hypoxanthine, and uracil for growth; Proto, prototrophic for proline, arginine, hypoxanthine, uracil, and methionine; Pro⁻, proline requiring.
§ MIC: Minimum inhibitory concentration of penicillin, expressed in micrograms per milliliter.

nor the convalescent sera were bactericidal for the homologous strain isolated from the affected patient. In two instances (1385 and 1349) the homologous strain was killed by the acute sera in titers of 8 and 32 and by the convalescent sera in titers of 16 and 64, respectively. In both cases the acute sera were obtained 4 or 5 days after onset of symptoms, before antibiotic therapy, and during a period of bacteremia as determined by concurrently positive blood cultures. One of these patients (1349) had biopsy-proven chronic active hepatitis. Each of the six strains from patients with DGI was resistant (titer < 2) to the bactericidal action of normal human sera (Table VI).

Complement activity of sera from patients with DGI. The total hemolytic complement activity of sera was determined for 18 patients with gonococcal arthritis at the time of their initial presentation. CH_{50} U/ml ranged from 78 to 172 (normal range 80–160), with a median of 123.

DISCUSSION

Gonococci were maintained as colony variants T1 or T2 for these experiments because these variants predominate on initial cultures from clinical material (16), have produced experimental urethritis in man (17) and chimpanzees (26–28), and are infective for the chick embryo (29, 30). In addition, they have been reported to be more resistant in an in vitro complement-dependent bactericidal assay than colony vari-

TABLE V

Relationship of Auxotype, Penicillin Susceptibility, and Serum Susceptibility of Cervical or Urethral Isolates Causing Uncomplicated Infection*

Penicillin G MIC§	Auxotype:‡	Number of strains with indicated penicillin MIC, auxotype, and serum susceptibility									
		Arg⁻Hyx⁻Ura⁻		Proto		Pro⁻		Arg⁻		Pro⁻Arg⁻	
		Serum resistant	Serum sensitive	Serum resistant	Serum sensitive	Serum resistant	Serum sensitive	Serum resistant	Serum sensitive	Serum resistant	Serum sensitive
2.0							1				
1.0				1	1	1					
0.50					6	1	5				
0.25				3	2	2		1			
0.125				1							1
0.060				1				1			
0.030		3			1	1					
0.015		9			1						
0.008											
0.004											
Total		12		6	11	5	6	2			1

* Serum susceptibility: Resistant, serum bactericidal titer <2; Sensitive, serum bactericidal titer ≥2.
‡ Auxotype: Arg⁻Hyx⁻Ura⁻, requires arginine, hypoxanthine, and uracil for growth; Proto, prototrophic for proline, arginine, hypoxanthine, uracil, and methionine; Pro⁻, proline requiring; Arg⁻, arginine requiring; Pro⁻Arg⁻, requires proline and arginine.
§ MIC: Minimum inhibitory concentration of penicillin G, expressed in micrograms per milliliter.

TABLE VI

Bactericidal Titer of Acute and Convalescent Sera from Six Patients with DGI for the Homologous Infecting Strains and Two Urethral Isolates from Uncomplicated Gonorrhea*

Sera		Strains							
		Disseminated gonococcal infection						Uncomplicated urethritis	
		1896	1939	1385	1950	1349	883	F62	6366
Pooled Normal human sera		<2	<2	<2	<2	<2	<2	4096	64
DGI sera (Days after onset of arthritis)									
1896	(2)	<2						4096	64
1896	(33)	<2						8192	64
1939‡	(6)		<2					2048	128
1939	(63)		<2					8192	128
1385‡	(4)			8				2048	16
1385	(27)			16				2048	16
1950	(8)				2			4096	128
1950	(44)				2			8192	256
1349‡	(5)					32		8192	32
1349	(29)					64		8192	16
883	(1)						2	2048	32
883	(13)						<2	4096	32

* Titer: Reciprocal of serum dilution resulting in ≥75% reduction in CFU compared to heat-inactivated serum control.

‡ Positive culture from blood or skin pustule on day acute serum was obtained.

ants T3 or T4 (31). Organisms were harvested in early log phase for use in the bactericidal assay since the rate of killing may be influenced by the bacterial growth phase (32, 33). The number of organisms in the reaction mixture was kept small ($\sim 5 \times 10^3$ CFU/ml) to minimize clumping during the assay, and the effect of clumping was controlled for by calculating percent survivors from a heat-inactivated serum control. Unabsorbed serum from a patient with X-linked agammaglobulinemia was used as the complement source because other complement sources are bactericidal for some gonococci and require absorption of bactericidal antibodies with gonococci. This process may result in the retention of soluble bacterial antigens that could inhibit the bactericidal reaction.

Only one of four serum-resistant strains maintained as colony variants T1 or T2 became more serum sensitive after 10 in vitro passes in the present study. However, Ward et al. (34), in a bactericidal assay uncontrolled for bacterial growth phase or colony type, found that gonococci in urethral exudates or grown on prostatic extract media (35) were serum resistant and that some became sensitive when retested after subculture on Kellogg's media.

Normal human sera from different donors tested against individual strains of serum-sensitive or resistant gonococci resulted in similar bactericidal titers (Table II). Though Abdoosh (36) was unable to demonstrate the presence of bactericidal antibody from normal human sera for three strains of gonococci, this study showed that most individuals without a history of gonorrhea appear to possess bactericidal antibodies against many gonococci isolated from uncomplicated infections. The origin of these naturally occurring bactericidal antibodies is unknown. It is unlikely that they arise as a result of prior infection with, or asymptomatic carriage of, the homologous species, although it has been shown that "natural" meningococcal antibodies can occur in this fashion (37, 38). It is possible they derive from the presence of cross-reactive antigens shared with meningococci (10, 39) or with nonpathogenic *Neisseria* present as respiratory tract commensals (36), or with enteric bacteria in a relationship analogous to the immunochemical similarity which exists between antigens of some species of *Escherichia coli* and the capsular polysaccharides of *N. meningitidis*, *Haemophilus influenzae* type B, and *Streptococcus pneumoniae* (40–44).

No sex-related differences in the bactericidal action of normal human sera were observed, although recent studies have shown DGI to be more common in women than in men (1). No relationship between blood type and the bactericidal power of sera was noted in this survey of serum from 20 normal donors,

though blood group antigens have been shown to cross react with those of some gram-negative organisms (45, 46) and an association between blood group and infections caused by enteric bacilli has been shown in several studies (47).

Significant differences were noted in the susceptibility of serum-sensitive strains to the bactericidal action of normal human sera (Table II). This was reflected in both the bactericidal titer and rate of killing of individual isolates such that a spectrum of serum susceptibility emerged as a continuum from nearly resistant to extreme sensitivity.

With a single exception, all strains isolated from patients with DGI were resistant to the bactericidal action of individual or pooled normal human sera from 20 male and female donors without a history of gonorrhea (Table IV). Spink and Keefer (11, 48) examined the complement-dependent bactericidal activity of whole defibrinated blood from uninfected normal controls and from individuals with localized or systemic gonococcal infections. In a standardized bactericidal assay, they noted that undiluted defibrinated blood from uninfected controls was able to kill ≥ 100 organisms from an unspecified inoculum for 7 of 16 isolates from individual patients with urethritis compared with 3 of 13 isolates from individual patients with gonococcal urethritis ($\chi^2 = 0.60, P > 0.10$). Although these differences did not achieve statistical significance, they postulated that the bactericidal activity of blood from normal individuals tended to be reduced for isolates from patients with systemic gonococcal infection, thereby predisposing to tissue invasion by these strains. They did not determine the titer of bactericidal antibody to systemic isolates in normal human serum, and their studies subsequently received little attention.

The mechanism for the observed variation in serum susceptibility of the strains studied here is unknown. It is of interest that two of six strains causing DGI, although resistant to the bactericidal action of normal human sera, were killed in low titer by the homologous convalescent sera (Table VI). In addition, normal rabbit sera were bactericidal in titers of 16 to 4,096 for six strains causing DGI which were resistant to the bactericidal action of normal human sera.[2] This may suggest that bactericidal antibodies to serum-resistant strains are unusual in normal human sera but common in normal rabbit sera. Alternatively, differences between serum-sensitive and serum-resistant strains may reside in quantitative differences in or the availability of bactericidal-reactive antigens (49). While the immunochemical characterization of these antigens is incomplete, Glynn and Ward (10) found that the antigens involved in the normal serum bactericidal reaction were resistant to heat and trypsin, and concluded that these antigens were lipopolysaccharides. Our own unpublished studies confirm their findings. Finally, it is possible that nonbactericidal antibody of the IgA class may compete for specific antigenic sites on serum-resistant strains, thereby inhibiting the action of bactericidal antibody (50).

A relationship between virulence and resistance to bactericidal antibody has been previously demonstrated for other gram-negative organisms (5, 6). Roantree and Rantz (7) found that bacteremic strains of enteric bacilli were serum resistant in 17 of 21 cases, but only 21 of 55 strains from stool and 7 of 30 strains from urine were resistant. Vosti and Randall (8) noted that 48 of 55 strains of *E. coli* isolated from blood were serum resistant compared with 94 of 141 from stool and 66 of 97 from urine. Young and Armstrong (9) found that 42 of 46 blood culture isolates of *Pseudomonas aeruginosa* were serum resistant compared with 34 of 43 "saprophytic" isolates from skin, mucous membranes, and gastrointestinal tract. These studies suggest that the bactericidal activity of serum may prevent the systemic invasion and survival of bacteria which are serum sensitive.

The bactericidal activity of normal serum for gram-negative organisms is complement dependent (6) and individuals with a deficiency or abnormality of certain complement components have an increased susceptibility to bacterial infections. In those with altered or diminished C3 or C5 activity, opsonization as well as bactericidal activity may be impaired (51–55), and susceptibility to gram-negative bacteria appears to be increased. Of particular interest is the observation that C6- and C8-deficient patients appear uniquely susceptible to bacteremic gonococcal or meningococcal infections (56–58), but usually not to other bacterial infections. These individuals possessed normal complement-dependent chemotaxis and opsonization, but lacked bactericidal activity for *N. gonorrhoeae* in one instance (57) and for *Salmonella typhi* and *H. influenzae*, type B in another (56). However, deficiency of C6 or C8 appears to be a relatively uncommon factor predisposing to gonococcal arthritis in Seattle, since CH_{50} levels of 18 patients presenting with gonococcal arthritis were determined, and were found to be normal. Complement levels unfortunately were not determined for the one patient with chronic arthritis, positive latex fixation test, and hyperglobulinemia, whose isolate was not serum resistant.

The bactericidal activity of acute and convalescent sera from patients with DGI was equal or superior to that of pooled normal human sera for two strains

[2] Schoolnik, G. K. Unpublished observations.

of serum-sensitive gonococci isolated from patients with uncomplicated gonorrhea (Table VI). It is therefore significant that patients with DGI do not appear to differ from those with localized gonococcal infections with regard to possession of bactericidal serum antibody to *N. gonorrhoeae*.

In contrast, disseminated *N. meningitidis* infection is also associated with the absence of bactericidal serum antibodies for epidemic strains, but the absence of bactericidal antibody is thought to be an unusual occurrence limited to a minority of adults. Goldschneider et al. found during a localized outbreak of meningococcal disease that individuals possessing bactericidal antibodies to the epidemic strain frequently became colonized by the organism, but rarely developed clinical disease (38).

Acute and convalescent sera from four of six patients with DGI were not bactericidal for the homologous infecting strain (Table VI). Two patients with DGI possessed bactericidal antibody for their strains and the bactericidal activity of the convalescent sera was enhanced for each by twofold compared to the acute sera. Spink and Keefer (11) observed an increase in bactericidal activity for some patients during the course of gonococcal arthritis, but their report did not indicate the proportion of patients showing such a rise. Buchanan et al. (59) found that each of seven patients with DGI showed high antibody levels or a rise in serum antibody to gonococcal pili, and Hess et al. (60), with an indirect fluorescent antibody method, noted antibody to gonococci in 100% of sera obtained 8–21 days after the onset of gonococcal arthritis. The absence of detectable bactericidal activity to the homologous strain in the convalescent sera of four of six patients in the present study is unexplained. This could be due to early treatment in this group of patients. Alternatively, the antigens involved in the bactericidal reaction may be less immunogenic than pili antigens or those antigens mediating immunofluorescence, or the sensitivity of the bactericidal reaction may be less than that of other tests for antibody to gonococci which cause DGI.

Although bacteremia was not observed in the presence of bactericidal antibody by Keefer and Spink (61), acute sera from two DGI patients in the present study were bactericidal in low titer for the homologous strain at the time of positive blood cultures. If the occurrence of gonococcemia can be confirmed in a larger number of patients whose serum is bactericidal, this would suggest that bactericidal antibody and complement alone do not prevent gonococcal bacteremia.

The fact that host factors other than bactericidal antibody may also be important in the pathogenesis of DGI is apparent from the following analysis. Strains requiring arginine, hypoxanthine, and uracil for growth were susceptible to ≤ 0.030 μg of penicillin G/ml and accounted for 98% of DGI and 38% of uncomplicated gonococcal infections in Seattle during 1971–1974 (3). 36 such isolates from patients with DGI were serum resistant (Table IV), as were each of 12 such isolates from patients with uncomplicated gonorrhea (Table V). If the estimate is accurate that 1–3% of Seattle patients with gonorrhea develop DGI (1), it follows that only about 3–8% of individuals infected with $Arg^-Hyx^-Ura^-$ serum-resistant strains develop systemic disease. Menstruation and pharyngeal infection are host factors which appear to favor the dissemination of serum-resistant strains (1, 2); however the host factors which prevent their dissemination are largely unknown.

Virulence factor(s) other than resistance to the bactericidal action of normal human sera appear to be important in the pathogenesis of DGI. Strains not requiring arginine, hypoxanthine, and uracil were isolated from approximately 62% of patients with uncomplicated gonorrhea in Seattle during 1971–1974 (3). In the present study, 13 of 31 (41.9%) of such strains were serum resistant (Table V). Thus, serum-resistant strains not requiring arginine, hypoxanthine, and uracil accounted for an estimated $41.9 \times 62 = 26\%$ of isolates from uncomplicated infections, but only 2% of strains causing DGI. Serum-resistant strains which require arginine, hypoxanthine, and uracil, may thus possess virulence factor(s) not present in serum-resistant strains with other auxotrophic requirements. These factor(s) are at present unknown and it is not clear whether they are linked to the $Arg^-Hyx^-Ura^-$ phenotype or are coincidentally associated in a clone of gonococci which is particularly common in Seattle. Similarly, the concurrence of the $Arg^-Hyx^-Ura^-$ auxotype, penicillin sensitivity, serum resistance, and the association with DGI has thus far only been documented for Seattle isolates. It is not known if it exists for strains from other geographic areas.

In conclusion, this study has established the significance of gonococcal resistance to the bactericidal action of normal human serum in the pathogenesis of DGI. However, serum resistance must be regarded as a necessary but not a sufficient condition for dissemination in patients with normal complement activity, both with respect to host factors (which may either favor or prevent dissemination of serum-resistant strains), and to virulence factor(s) other than serum resistance. The phenotypic association of the $Arg^-Hyx^-Ura^-$ auxotype, penicillin sensitivity, serum resistance, and capacity to cause DGI has been documented for Seattle isolates and its possible epidemiologic, genetic, and physiologic bases and the

immunochemical substrate of serum resistance remain intriguing problems requiring further study.

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