Gonococcal Color and Opacity Variants: Virulence for Chicken Embryos

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Previous studies have noted that the prevalence of certain gonococcal colony types is influenced by the physiological state of the human host. Consequently eight different gonococcal strains were passed on clear typing medium, and opaque (Op) and transparent (Tr) variants of heavily pilated (P++) bacteria were selected. P++Op and P++Tr were injected into 11-day-old chicken embryos, and the resulting mortality was determined. In every case the Tr variants were more lethal to the embryos than were the Op variants ($P = 0.01$). P++Tr were stable *in vivo*, but chicken embryos injected with P++Op strains developed bacteremia with P++Tr organisms. However, chicken blood was not bactericidal for either colonial variant. These results indicate that transparent pilated bacteria may be more virulent than opaque pilated bacteria and that *in vivo* selection of transparent forms occurs.

The classification of gonococci by Kellogg et al. (13, 14) into four colony types initiated much investigative work into the pathogenetic mechanisms of this bacterium. It has been recognized for some time, however, that not all gonococcal colonies can be encompassed by this typing system (2, 6, 9). Recently, Swanson has studied gonococcal colonial variants which have similar edge morphology to that described by Kellogg but which include a wider variation in opacity and color (21). This expanded classification is based on three seemingly independent characteristics: presence or absence of piliation, colonial color (ranging from fawn to black), and colonial opacity (ranging from transparent to opaque). Swanson found the opaque bacteria to be more aggregated than the transparent forms, and the former were also more sensitive to killing by trypsin (12, 21).

The role of these bacterial variants in pathogenesis of gonococcal infections is unknown. James has found a predominance of darker, more opaque colonies in cultures from male urethras and from cervical cultures of women who were near the midpoint of their menstrual cycle (12). More of the transparent, light, and trypsin-resistant colonies were isolated around the time of the menses, and it is at this time that salpingitis and disseminated gonococcal disease occur most commonly.

Because the virulence of other bacteria can be predicted by differences in colonial opacity (7, 16), we tested the virulence of gonococcal color

variants by determining their lethality for 11-day-old chicken embryos.

MATERIALS AND METHODS

Gonococci and medium. Strains F62 and 33 have been passed in our laboratory for several years. All other isolates were obtained from Cornell University Medical School, Department of Microbiology. Isolates were passed on a gonococcal typing medium as previously described (21).

Colonies were classified based on their appearance on agar as viewed through a Stereo Zoom 7 dissecting microscope with photographic attachment (Bausch & Lomb, Inc., Rochester, N.Y.). Colony edge morphology and color were assessed by using the diffusing surface of the substage reflector, and the plane polished mirror was used to judge opacity. Twitching motility was observed using a Zeiss photomicroscope. Colonial highlights (19) were assessed through a dissecting microscope with an elevated light source at an angle of 45° above the horizontal.

A single lens reflex camera fitted to the dissecting microscope was used to photograph the colonial types. Kodak Panatomic film (ASA 32) was used with an exposure of 0.5 s and an initial magnification of $\times 20$.

For experiments, a single colony was selected which possessed the desired edge morphology, coloration, and opacity. From each gonococcal strain, one colony type was selected which was opaque (Op), dark brown to black in color, and contained heavily pilated bacteria (P++) and another was selected which was transparent (Tr), yellow to light brown in color, and also contained heavily pilated bacteria (P++). From some strains, nonpilated variants were also selected. The appropriate colony was streaked on an agar plate and grown for 18 to 20 h at 37°C in a candle jar at which point the colonial morphology was reconfirmed. Bacteria were removed from the agar by cotton-tipped

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swabs and were suspended in proteose peptone-saline (pH 7.4) (proteose peptone no. 3, 10 g/liter; sodium chloride, 5 g/liter) in polystyrene tubes (16 by 125 mm) to an optical density of 0.1 at a wavelength of 540 nm using a Coleman model 44 linear absorbance spectrophotometer (Perkin-Elmer Corp., Maywood, Ill.). This corresponded to 3×10^7 to 4×10^7 colony-forming units (CFU) per ml for P++Op and 7×10^7 to 9×10^7 CFU per ml for P++Tr. Serial dilutions were made in proteose peptone-saline, and the viable counts of the bacteria for injection were determined for each experiment.

Chicken embryos. Eleven-day-old embryos were obtained from White Leghorn flocks which had been fed antibiotic-free meal. The embryos were stored in a humidified incubator at 37°C and were prepared for intravenous inoculation by using the method of Eichhorn (10). Five concentrations of bacteria of each colonial type were prepared in proteose peptone-saline, and 100 μ l was injected intravenously by using at least six embryos for each dilution. We discarded embryos which died or had excessive bleeding from the injection site when examined at 2 to 3 h. Assessment of mortality was made by candling the remaining eggs at 24 and 48 h after injections. Blood cultures were done by removing the shell over the air sac and nicking a blood vessel with a 23-gauge needle. The blood was collected with sterile micropipettes and was diluted in proteose peptone-saline for quantitation on agar plates and for assessment of colonial types.

The bactericidal effect of chicken blood was determined by collecting blood as described above from uninoculated embryos and pooling the samples. Heparin and known amounts of bacteria were added to the blood in 1.5-ml microfuge tubes, and the mixture was rotated end-over-end at 37°C. Aliquots were taken at 30, 60, and 90 min, and the numbers of viable bacteria and colony types were determined.

RESULTS

Lethality of opacity variants. Op and Tr variants of colonies with very sharply defined margins (T2 edge morphology) were used for most experiments (Fig. 1). These Op and Tr variants were determined to be heavily pilated (P++) by electron microscopy, and similar amounts of pili have been isolated from both types. (I. E. Salit, M. Blake, and E. C. Gotschlich, manuscript submitted for publication). P++Tr colonies demonstrated twitching motility, and P++Op did not. Both colony types exhibited double highlights as demonstrated by reflected light.

Dilutions of P++Op and P++Tr from one strain were injected on the same day into the same batch of chicken embryos. Eight different strains of gonococci were studied in this manner (Table 1). In every instance, the 50% lethal dose (LD_{50}) at 24 or 48 h of P++Tr was less than the corresponding LD_{50} of the P++Op derived from the same strain ($P = 0.01$, Wilcoxon signed rank test). Injections were repeated on different days

for all strains: the LD_{50} was reasonably reproducible, and in all cases the LD_{50} of Tr forms was lower than that of the Op. More chicken embryos were dead at 48 h compared to 24 h with both opacity variants, but the LD_{50} of P++Tr remained about 10 times less than that of the P++Op at both time points (ratio at 24 h was 9.9 and at 48 h was 11.8).

Stability of colony types. During in vitro passaging on typing agar, both pilated and non-pilated opaque colonial forms tended to change to transparent colonies at a frequency of about 1 to 2%. Transparent colonial types rarely reverted to opaque forms. Thus, P++Op bacteria for injection were 98 to 99% Op (1 to 2% were P+ or P++Tr, and less than 0.5% were P-Op), and samples of P++Tr for injection were 100% Tr (2 to 4% were P-Tr).

Colonial survival and stability were excellent on agar and in liquid media of similar composition, but markedly different in the chicken embryos. After injection of about 10^5 CFU of P++Op or P++Tr, the mortality was 95 to 99% at 24 h. Quantitative blood cultures on similarly injected embryos (Fig. 2) revealed that by 4 h, greater than 10-fold more viable bacteria could be harvested from the eggs injected with P++Tr compared to those injected with P++Op. Similar differences were present at 8 and 10 h. The three strains which were studied (F62, R16, and MS-11) gave very similar results except that F62 and MS-11 had a 2- to 10-fold decrease of viable bacteria within the first hour.

We then determined the colony types remaining in the chicken bloodstream after injection of P++Op or P++Tr. Quantitative blood cultures up to 10 h after injection of P++Tr indicated that all the bacteria were transparent (over 90% were P+ or P++ and less than 10% were P-). Similar cultures from embryos injected with P++Op (Fig. 3) showed that the numbers of Op bacteria in the bloodstream diminished rapidly after 1 h so that by 8 h more than 99% of the remaining bacteria were Tr. It was these Tr bacteria which then multiplied in vivo (Fig. 4).

Several experiments were done to determine the relative virulence of Tr and Op nonpilated (P-) gonococci for the 11-day-old chicken embryos. The LD_{50} of P- bacteria was more than 300 times higher than the average of those found for the P++ bacteria. For each of these strains tested, the LD_{50} of the P-Op and P-Tr did not differ by more than 30%. Quantitative blood cultures also indicated that P-Tr were selected for in vivo, but to a lesser degree than pilated forms.

Bactericidal effect of chicken embryo blood. Pooled fresh heparinized blood had no

FIG. 1. Typical examples of bacterial colonies used in this study. *R16 P++Op* (A, B) and *R16 P++Tr* (C, D) as viewed with diffuse transmitted light to demonstrate color intensity (A, C), and with light transmitted from the plane polished mirror to demonstrate opacity (B, D).

effect on the viable counts of *P++Op* and *P++Tr* after incubations up to 90 min, and no change in colonial type was present after these incubations. Results were similar after incubating the bacteria in fresh chicken plasma. In addition, prefilter disks soaked with 150 μ l of chicken blood were placed on freshly inoculated agar plates. After 24 h of incubation, no zones of inhibition were seen, although, for some strains, we could demonstrate zones of inhibition when trypsin was applied to the disk (12).

Bacterial capsules. Bacteria stained with methylene blue and India ink demonstrated definite halos. Although the size of the clear areas varied from strain to strain, no definite differences were seen between *Op* and *Tr* bacteria or in *Tr* bacteria before and after inoculation into chicken embryos.

DISCUSSION

Intravenous inoculation of 11-day-old chicken embryos has been used previously as a model for gonococcal (5, 6, 8), meningococcal (4), and streptococcal (24) infections. Colonial types of gonococci which have proven to be virulent for human volunteers (13) and chimpanzees (15) are considerably more lethal than the avirulent gonococcal colony types when injected intrave-

nously into chicken embryos (5, 6, 8). Virulent gonococci are also more invasive as measured by passage across the chorioallantoic membrane into the allantoic fluid (3). Such studies indicate that the 10- to 11-day-old chicken embryo may be a useful model for the assessment of pathogenic potential of gonococcal colony types. Previous studies in this model have mainly compared the virulence of the four colony types described by Kellogg. Chan and Wiseman (6) did examine a pilated opaque variant of T1 (type 1¹) and determined that it was virulent for chicken embryos. A systematic comparison of the virulence of opacity variants was not made, however.

Swanson and James (12, 21) have recently reported the common occurrence of gonococcal colonial forms that do not fall into the previously accepted categories. Their studies have led to the recognition of three probably independent characteristics of gonococcal colonies: presence or absence of pilation (which is correlated with colony size and edge morphology), color, and opacity. Colonies which are opaque (usually also dark brown to black) are sensitive to killing by trypsin and are found predominantly in cervical exudates from infected females who are near the midpoint of their menstrual cycle. Lighter col-

TABLE 1. Virulence for 11-day-old chicken embryos of opacity variants of heavily pilated gonococci

Strain	LD ₅₀ at 24 h ^a		LD ₅₀ at 48 h	
	Op	Tr	Op	Tr
33	9,000	750	4,670	91
	1,720	102	314	78
MS-11	1,200	184	600	107
	790	144	102	74
F62	36 ^b	<10	20	<10
	607 ^c	67	98	32
	720 ^c	110	489	54
R01	81	<20	ND ^d	ND
	210	10	ND	ND
R10	320	74	55	29
	260	60	30	12
R12	3,640	328	473	<10
	1,840	260	360	120
R16	1,944	80	86	<12
	488	200	231	<9
R17	671	72	110	16
	420	42	80	12

^a Calculated by the method of Reed and Muench (20).

^b Extra-dark variant.

^c Dark variant.

^d ND, Not determined.

FIG. 2. Results of quantitative blood cultures after injection of strain R16. The two curves depict total viable bacteria of any type remaining in the bloodstream after injection of (▲) P++Tr and (■) P++Op. Symbols on the ordinate are total numbers of bacteria injected. Each point represents the arithmetic mean of the results obtained with seven injected embryos.

ored, transparent colonies are insensitive to trypsin and are isolated predominantly around the time of the menses. In addition, cultures

from male urethras tend to contain darker, more opaque colonies, whereas women taking oral contraceptives tend to have light, transparent colonies throughout the menstrual cycle.

It was because of these findings that we decided to determine the lethality of the opacity variants for the chicken embryo. We predominantly studied differences in embryo mortality after injection of pilated gonococci because it is these bacteria which are found in clinical specimens. In the eight different strains investigated, the transparent, light colony types were more virulent than the opaque, dark colony types. The fact that the Op bacteria are capable of killing the embryos at all appears to be due to the small numbers of Tr bacteria inevitably injected at the same time. A less likely possibility

FIG. 3. Colonial types remaining in the embryo bloodstream after injection of R16 P++Op. Symbols on the ordinate are total numbers of opaque (●) and contaminating transparent (○) bacteria injected. Each data point is derived from seven injected embryos. At 10 h (*) no Op bacteria could be found.

Stability of Colony Types *in Vivo*

FIG. 4. Percentage of colonial types remaining in the chicken embryo bloodstream after injection of P++Op. The results are from three separate experiments with strains R16, MS11, and F62. □, Translucent colonies; ■ opaque colonies.

is that the Op bacteria became converted to a Tr morphology in vivo. Whatever mechanism may be operative, it is apparent that by 10 h after injection, the Op bacteria are no longer found in the chicken bloodstream but are replaced by Tr bacteria, which multiply in number. When Tr bacteria alone are injected they do not change colonial morphology and they do kill the embryos. It is thus assumed that the P++Tr forms are responsible for the chicken embryo mortality even after injection of P++Op forms.

We were not able to demonstrate that the shift in colony type was due to proteolytic activity that may be lethal to Op forms since chicken blood was not bactericidal for the pilated or nonpilated opacity variants. Bumgarner and Finkelstein (5) also found no bactericidal activity of chicken blood for T1 and T3 colony types.

The mechanism for the differences in virulence between P++Op and P++Tr forms for the chicken embryo is unknown. These experiments do not negate the importance of pili as a virulence factor since P++ bacteria possessing extremes of opacity or color were considerably more virulent than P- bacteria of similar color and opacity. We have purified pili from the Op and Tr variants of all strains used in this study and have often found physicochemical differences between pili from Op and Tr bacteria of the same strain. (I. E. Salit et al., manuscript submitted for publication). We usually isolated the same amount or more pili from the Op bacteria than from the Tr forms, so it is unlikely that differences in numbers of pili per bacteria could explain the enhanced virulence. However, other differences in protein composition have also been found when the outer membranes of Op and Tr bacteria are compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22). In some bacterial species, variation in colony opacity can be a marker for differences in the lipopolysaccharide of the outer membrane (1), but this has yet to be explored for gonococci. Other possible explanations for enhanced virulence associated with opacity variation may be altered iron chelation (18), perhaps correlation with the leukocyte association factor (23), or differences in encapsulation. However, we found no differences in sizes of peribacterial halos between Op and Tr bacteria stained with India ink.

Opacity variations in other bacteria can also be a marker for virulence. Cooper et al. (7) found some Op forms of a *Shigella flexneri* strain to be avirulent for mice although the Tr forms were virulent. This Tr form was subsequently shown to be more virulent when fed to monkeys and guinea pigs probably because of enhanced invasiveness (16).

Opacity and color variation of colonies can thus be a marker for antigenic variation (11, 17) and virulence and is of importance in selecting appropriate bacteria for immunization studies. If such variants of the gonococcus exhibit similar differences in virulence for human beings, one could speculate that predominance of Tr forms around menstruation may be responsible for the increased incidence of complications at this time.

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LITERATURE CITED

1. Braun, W. 1966. Representative types of mutants. III. Mutations affecting colonial morphology, p. 153-163. *In* Bacterial genetics. The W. B. Saunders Co., Philadelphia.
2. Brown, W. J., and S. J. Kraus. 1974. Gonococcal colony types. *J. Am. Med. Assoc.* 228:862-863.
3. Buchanan, T. M., and E. C. Gotschlich. 1973. Studies of gonococcus infection. III. Correlation of gonococcal colony morphology with infectivity for the chick embryo. *J. Exp. Med.* 137:196-200.
4. Buddingh, G. J., and A. D. Polk. 1939. Experimental meningococcus infection of the chick embryo. *J. Exp. Med.* 70:485-497.
5. Bumgarner, L. R., and R. A. Finkelstein. 1973. Pathogenesis and immunology of experimental gonococcal infection: virulence of colony types of *Neisseria gonorrhoeae* for chicken embryos. *Infect. Immun.* 8:919-924.
6. Chan, K., and G. M. Wiseman. 1975. A new colonial type of *N. gonorrhoeae*. *Br. J. Vener. Dis.* 51:251-256.
7. Cooper, M. L., H. M. Keller, and E. W. Walters. 1957. Microscopic characteristics of colonies of *Shigella flexneri* 2a and 2b and their relation to antigenic composition, mouse virulence and immunogenicity. *J. Immunol.* 78:160-171.
8. Diena, B. B., G. Laverne, A. Ryan, F. E. Ashton, R. Wallace, M. Perry, and V. Daoust. 1975. The chick embryo in studies of virulence and immunity with *Neisseria gonorrhoeae*. *Rev. Can. Biol.* 34:213-220.
9. Diena, B. B., A. Ryan, F. E. Ashton, and R. Wallace. 1974. Colonial morphology of *Neisseria gonorrhoeae*. Letter to the Editor. *J. Am. Med. Assoc.* 229:1422.
10. Eichhorn, E. 1940. A technique for the intravenous inoculation of chick embryos. *Science* 92:245-246.
11. Enomoto, M., and T. Iino. 1963. Colonial dimorphism in nonmotile *Salmonella*. *J. Bacteriol.* 86:473-477.
12. James, J. F., and J. Swanson. 1978. Studies on gonococcus infection. XIII. Occurrence of color/opacity colonial variants in clinical cultures. *Infect. Immun.* 19:332-340.
13. Kellogg, D. S., Jr., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reising. 1968. *Neisseria gonorrhoeae*. II. Colonial variation and pathogenicity during 35 months in vitro. *J. Bacteriol.* 96:596-605.
14. Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *S. Bacteriol.* 85:1274-1279.
15. Kraus, S. J., W. J. Brown, and R. J. Arko. 1975. Acquired and natural immunity to gonococcal infection

- in chimpanzees. *J. Clin. Invest.* **55**:1349-1356.
16. **Labrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. *J. Bacteriol.* **88**:1503-1518.
 17. **Nicolle, P., A. Jude, and L. Le Minor.** 1950. Relation entre l'intensite de l'irisation presentee par certain colonies de Salmonella et leur constitution antigenique. *Ann. Inst. Pasteur (Paris)* **78**:527-582.
 18. **Payne, S. M., and R. A. Finkelstein.** 1975. Pathogenesis and immunology of experimental gonococcal infection: role of iron in virulence. *Infect. Immun.* **12**:1313-1318.
 19. **Penn, C. W., D. R. Veale, and H. Smith.** 1977. Selection from gonococci grown *in vitro* of a colony type with some virulence properties of organisms adapted *in vivo*. *J. Gen. Microbiol.* **100**:147-158.
 20. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**:493-497.
 21. **Swanson, J.** 1978. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. *Infect. Immun.* **19**:320-331.
 22. **Swanson, J.** 1977. Surface components associated with gonococcal-cell interactions, p. 369-401. *In* R. Roberts, ed.), *The gonococcus*. John Wiley & Sons, New York.
 23. **Swanson, J., E. Sparks, D. Young, and G. King.** 1975. Studies on gonococcus infection. X. Pili and leukocyte association factor as mediators of interactions between gonococci and eukaryotic cells *in vitro*. *Infect. Immun.* **11**:1352-1361.
 24. **Tieffenberg, J., L. Vogel, R. R. Kretschmer, D. Padnos, and S. P. Gotoff.** 1978. Chicken embryo model for type III group B beta-hemolytic streptococcal septicemia. *Infect. Immun.* **19**:481-485.