STUDIES ON GONOCOCCUS INFECTION

III. CORRELATION OF GONOCOCCAL COLONY MORPHOLOGY WITH INFECTIVITY FOR THE CHICK EMBRYO*

BY THOMAS M. BUCHANAN AND EMIL C. GOTSCHLICH

(From The Rockefeller University, New York 10021)

(Received for publication 26 October 1972)

In 1963 Kellogg et al. classified gonococci into four types on the basis of colonial morphology (1). He and his co-workers further observed that types 1 and 2 produced gonorrhea in eight of eight human volunteers inoculated intraurethrally, whereas none of eight volunteers receiving comparable numbers of types 3 and 4 organisms developed disease (1, 2). This correlation between colony type and virulence has not been tested in an animal model.

The chorioallantoic membrane of chick embryos was infected with gonococci by Morrow and Berry in 1938 (3), and subsequently by Hill and Pitts (4) and Bung (5). Our approach has been to use this convenient and inexpensive model to investigate differences in virulence of the four gonococcal colony types.

Materials and Methods

Strains of N. gonorrhoeae.—The four colony types from each of two strains of N. gonorrhoeae F62 and 2868 were used. All eight bacterial lines had been subcultured many times to maintain distinct colonial morphology. One of the strains (F62) was originally used by Kellogg et al. to compare different colony types for their infectivity to human volunteers (1). Cultures were grown on gonococcal agar base supplemented with Isovitalex (Baltimore Biological Laboratories, Baltimore, Md.) and were preserved by freezing in a medium consisting of 5% wt/vol bovine serum albumin and 5% wt/vol monosodium glutamate (6).

Chick Embryo Technique.—10-day old embryonated eggs were obtained from White Leghorn flocks which had been fed antibiotic-free meal. The chorioallantoic membrane was exposed according to the method of Beveridge and Burnet (7). Known numbers of viable gonococci in 50 μl of phosphate-buffered saline were dropped onto the intact chorioallantoic membrane. The opening of the shell was resealed with a cover slip and paraffin and the eggs were incubated at 36.5°C. After 2 days, a sterile needle and syringe were used to pierce the chorioallantoic membrane and withdraw 0.75 cc of chorioallantoic fluid, which was cultured on Thayer-Martin media (8). After adsorption of the fluid by the media, the plates were inverted and incubated at 36.5°C in a candle extinction jar. Individual colonies containing gram-negative diplococci were subcultured into Isovitalex-enriched gonococcal agar to determine colony type and onto cysteine trypticase agar to determine sugar fermentation patterns. Organisms which fermented glucose but not maltose were accepted as gonococci (9).

Quantitation of the inocula.—Gonococci of a given colony type grown for 18-20 h were suspended in phosphate-buffered saline (pH 7.2) using a Vortex Jr. mixer (Scientific Industries, Inc., Beverly, Mass.) and diluted to an optical density at 660 nm of 0.1. These suspensions were then diluted further to contain 5, 10, 20, or 50 viable gonococci per inoculum.

* Supported by U. S. Public Health Service grants 5F03-AI-50, 525-02, and AI10615-01.
Inc., Springfield, Mass.) and adjusted to an OD of 0.20 at 650 nm (Coleman Jr., Model 6A spectrophotometer, Coleman Instrument Div., Perkin Elmer Corp., Maywood, Ill.) in 16 X 125 mm clear plastic tubes. This suspension was sonicated for 2 s to disperse bacterial clumps with a Sonifier Cell Disruptor Model W 185 (Heat Systems-Ultrasonics, Inc., Branson Sonic Power Co., Danbury, Conn.) delivering 35 W/s. This period of sonication was chosen because it doubled the colony-forming units for T2 suspensions and produced no significant change in T1, T3, or T4 suspensions. Sonification for 5 s or longer resulted in cell death in all suspensions. Microscopic examination of suspensions sonicated for 2 s showed only diplococci and single cocal forms. The sonicated suspension (approximately $10^9$ organisms/ml) was diluted in medium 199 pH 7.2 (Grand Island Biological Co., Grand Island, N. Y.) to $10^6$-$10^7$ organisms/ml and the diluted suspension was used as the inoculum for the chick embryos. Plate counts were made in duplicate from the inoculating dilution, both before and immediately after inoculation of a group of chick embryos. No significant change in viable count was observed during the approximately 30 min required to infect a group of chick embryos.

**Blood Cultures and Histologic Studies.**—Blood was obtained from 41 chick embryos whose chorioallantoic fluid had yielded gonococci. Blood was removed from 18 infected chick embryos 4-6 days after inoculation and from 23 infected embryos at time of hatching. Approximately 0.1 cc of blood was obtained by cannulation of a vein in the direction opposite to the flow with a sterile syringe and 27 gauge needle, a modification of the method of Eichorn (10). The blood was streaked onto gonococcal agar base supplemented with Isovitalex and cultured at 36.5°C in a candle extinction jar. Isolates were identified as gonococci by sugar fermentations and gram stain.

11 chick embryos infected with gonococci and two embryos which had not been inoculated were fixed in 10% formalin, decalcified, and stained with hematoxylin and eosin or tissue gram stain. Sections of the chorioallantoic membrane, heart and heart valves, meninges, and joints were examined for evidence of gonococcal infection.

### RESULTS AND DISCUSSION

Our data and previous investigations by Bang (5) indicate that gonococci usually produce a localized infection of the chick embryo chorioallantoic membrane. In this study, only one of 18 blood cultures obtained from embryos 4-6 days after infection and none of 23 blood cultures obtained at the time of hatching yielded gonococci. This suggests that sustained gonococcal bacteremia is not a common accompaniment of gonococcal infection of the chorioallantoic membrane and cavity. Furthermore, none of 11 chicks examined histologically showed evidence of systemic infection. Nevertheless, it appears that gonococcal infection of the chick chorioallantoic membrane and cavity does contribute to death of the chick embryo. Of 128 chick embryos which developed a gonococcal chorioallantoic fluid infection and were observed to the time of hatching in this study, 71 or 55% died. Similarly, 32 of 71 (41%) embryos inoculated with gonococci, but whose chorioallantoic fluid was sterile when cultured, died before hatching. Controls included 20 eggs with a shell opening only, 20 with shell opening and 50 µl of sterile saline added but no chorioallantoic fluid removed, and 20 inoculated with 50 µl of sterile saline with subsequent removal of 0.75 cc of chorioallantoic fluid. Mortality rates among the controls were 0 of 20, 1 of 20, and 6 of 20, respectively. None of the controls were contaminated with bacteria at the time of death and six of the seven died just before hatching. Death in the
inoculated embryos occurred almost always 3-4 days after administration of gonococci, a time when inflammation and thickening of the chorioallantoic membrane is prominent (5). Chorioallantoic fluid cultured at the time of death from 46 of these chick embryos yielded in 43 instances *N. gonorrhoeae*, suggesting that gonococci may have contributed to the death. Our histologic observations and those of Bang (5) suggest that the contribution of gonococci to the death of embryos is through changes produced in the chorioallantoic space rather than by causing a systemic infection of the embryo.

Gonococcal urethritis and cervicitis in humans appears somewhat analogous to infection of the chick chorioallantoic membrane with *N. gonorrhoeae*. In both instances the infection is localized (5, 11), and in each infection the frequency of bacteremia is low (12, 13). Barr and Danielsson found that only 4 of 542 men (0.7%) and 16 of 532 women (3%) had evidence of sepsis with their gonococcal infection (12). Holmes et al. estimated that 0.6-1.1% of persons asymptptomatically infected with gonococci developed sepsis (13).

**TABLE I**

| Inoculum size | Type 1 | Type 2 | Type 3 | Type 4 |
|---------------|--------|--------|--------|--------|
|               | no. positive | no. total | no. positive | no. total | no. positive | no. total | no. positive | no. total |
| 1,000         | 8      | 14     | 0      | 5      | 0      | 5      |
| 1,000-19,999  | 8      | 14     | 8      | 14     | 1      | 22     |
| 20,000-200,000| 27     | 35     | 9      | 12     | 4      | 24     |
|              | 69% (52/75) | 11% (9/84) |

The infection rate by size and colony type of inoculum for 159 chick embryos infected with gonococcal strain 2868 is summarized in Table I. When less than 1,000 gonococci were administered no infection of the chorioallantoic fluid occurred, irrespective of colony type. However, when 1,000-200,000 organisms were inoculated, types 1 and 2 gonococci produced chorioallantoic fluid infection significantly more often (69%) than comparable numbers of colony types 3 or 4 (11%, \( P < 0.001 \)). Differences in infection rate produced by type 1 as compared with type 2 organisms or by type 3 as compared with colony type 4 were not significant (Table I).

Table II summarizes the results of chorioallantoic fluid cultures for 77 chick embryos inoculated with gonococcal strain F62. Again, colony types 1 and 2 produced chorioallantoic fluid infection more often (68%) than colony types 3 and 4 (15%, \( P < 0.001 \)). No significant differences were observed between the infection rates produced by type 1 as compared with type 2, or between type 3 as compared with type 4 organisms with the F62 strain.

The demonstration that gonococcal colony types 1 and 2 are more infective than types 3 and 4 for both man (1, 2) and the chick embryo suggests some
TABLE II

| Inoculum size | Types 1 and 2 | Positive | Types 3 and 4 | Positive |
|---------------|--------------|----------|--------------|----------|
|               | no. positive | no. total | no. positive | no. total |
| 1,000-19,999  | 12           | 19       | 1            | 20       | 5        |
| 20,000-299,000| 13           | 18       | 72           | 5        | 25       |
| Total         | 25           | 37       | 68           | 20       | 15       |

fundamental differences between the colony types. Jephcott, Reyn, and Birch-Andersen (14) and Swanson, Kraus, and Gotschlich (15) have recently demonstrated that filamentous structures known as pili are present on types 1 and 2 organisms, but absent from types 3 and 4 gonococci. Whether these pili play a pathogenic role or are merely markers of other virulence factors remains to be determined.

The chick embryo is without complement or antibodies until shortly after hatching (7) and thus provides a model in which direct effects of the gonococcus and its interaction with phagocytic cells may be studied. It also provides a model in which to study the protective effects of passively administered antibody in an environment where antibody-cell interactions, with or without complement, can take place (16).

SUMMARY

Comparable numbers of types 1, 2, 3, and 4 gonococci were placed on the intact chorioallantoic membrane of 236, 10-day old chick embryos. Types 1 and 2 organisms produced infection and could be cultured from chorioallantoic fluid 2 days later significantly more often (69%) than types 3 and 4 organisms (12%, $P < 0.001$). This confirms in an animal model the same correlation between colony types and infectivity observed in human volunteers and suggests that types 1 and 2 gonococci possess a fundamental virulence characteristic which is absent from types 3 and 4 organisms. Gonococcal infection of the chick embryo chorioallantoic cavity remains a useful model somewhat analogous to localized gonococcal infection in man.

The authors are indebted to Mr. Leon Parkes for his able assistance in these studies.

REFERENCES

1. Kellogg, D. S., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle. 1963. Neisseria gonorrhoeae. I. Virulence genetically linked to clonal variation. J. Bacteriol. 85:1274.
2. Kellogg, D. S., 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.
3. Morrow, G., and G. P. Berry. 1938. The cultivation of Neisseria gonorrhoeae on the chorioallantoic membrane of the chick embryo and the use of this technique for the study of sulfanilamide. J. Bacteriol. 36:280.
4. Hill, J. H., and A. C. Pitts. 1939. The growth of Neisseria gonorrhoeae on the chorioallantoic membrane of the chick embryo. J. Urol. 41:81.
5. Bang, F. 1941. Experimental gonococcus infection of the chick embryo. J. Exp. Med. 74:387.
6. Greaves, R. I. H. 1960. Preserving living cells by freeze-drying. Ann. N.Y. Acad. Sci. 85:723.
7. Beveridge, W. I. B., and F. M. Burnet. 1946. The cultivation of viruses and Rickettsiae in the chick embryo. Med. Res. Coun. (G.B.) Spec. Rep. Ser. 266.
8. Thayer, J. D., and J. E. Martin, Jr. 1964. A selective medium for the cultivation of N. gonorrhoeae and N. meningitidis. Public Health Rep. 79:49.
9. Bodily, H. L., E. L. Updyke, and J. O. Mason, editors. 1970. Diagnostic Procedures for Bacterial, Mycotic, and Parasitic Infections. American Public Health Association, New York. 5th edition. 296.
10. Eichorn, E. A. 1940. A technique for the intravenous inoculation of chick embryos. Science (Wash. D.C.). 93:245.
11. Finger, E., A. Ghon, and F. Schlegenhauer. 1894. Beiträge zur biologie des gonococcus und zur pathologischen anatomie des gonorrhoeischen processes. Arch. Dermatol. Syph. 28:277.
12. Barr, J., and D. Danielsson. 1971. Septic gonococcal dermatitis. Br. Med. J. 1:482.
13. Holmes, K. K., P. J. Wiesner, and A. H. B. Pedersen. 1971. The gonococcal arthritis-dermatitis syndrome. Ann. Intern. Med. 75:470.
14. Jephcott, A. E., A. Reyn, and A. Birch-Andersen. 1971. Brief report: Neisseria gonorrhoeae. III. Demonstration of presumed appendages to cells from different colony types. Acta Pathol. Microbiol. Scand. Sect. B: Microbiol. Immunol. 79:437.
15. Swanson, J., S. J. Kraus, and E. C. Gotschlich. 1971. Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. J. Exp. Med. 134:886.
16. Buddingh, G. J., and A. D. Polk. 1939. A study of passive immunity to meningococcus infection in the chick embryo. J. Exp. Med. 70:511.