Effect of Estrogen (17β-Estradiol) on the Susceptibility of Mice to Disseminated Gonococcal Infection

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Studies of the effect of sex hormones on the susceptibility of mice to the disseminated gonococcal infection demonstrated significantly enhanced susceptibility of mice injected with estrogen (17β-estradiol). In mice treated with estradiol, bacteremia progressively developed within 12 h postinoculation and mice died within the next 6 h, whereas bacteremia in mice treated with progesterone was completely cleared within 3 h postinoculation. The administration of estradiol affected the function of polymorphonuclear leukocytes (PMN) responsible for eliminating gonococci, but the administration of progesterone did not. The bactericidal activity of PMN mediated by myeloperoxidase was affected by estradiol, but the capacity of PMN to release superoxide anion was not. Furthermore, peritoneal cell analysis demonstrated that the infiltration of PMN in the peritoneal cavity of estradiol-treated mice significantly decreased when mice were injected intraperitoneally with gonococci. These effects on PMN by estradiol may play an important role in the enhanced susceptibility of estradiol-treated mice to gonococcal infection.

Human hormonal variation during the menstrual cycle affects the host resistance against several microbial infections. Several investigators have reported that pregnancy increases the host susceptibility to fungal infections (10, 13), viral infections (2, 3, 15, 30), and bacterial infections (25) including disseminated gonococcal infection (DGI) (17).

Variation of host susceptibility associated with pregnancy was explained in part by immunological depression (5, 11, 20, 27, 29, 37) which was caused by hormonal changes. In pregnant women, the levels of both estrogen and progesterone in serum increase, and these sex hormones have various biological effects which could act in vivo directly or indirectly on microbes. In gonococcal infections, estrogen can stimulate the growth of gonococci in vitro, but progesterone inhibits such growth (31).

Our previous study (21) demonstrated that female mice were the most susceptible to genital gonococcal infection at the proestrous stage, at times when the estrogen level in serum was highest. Although it has been reported that pregnant women have an increased susceptibility to DGI (17), it is not known whether the enhanced susceptibility of pregnant women to DGI is due to the high level of estrogen in serum. Furthermore, the level of progesterone in serum capable of inhibiting gonococcal growth is also highest during pregnancy. Thus, the present study was undertaken to clarify the role of reproductive hormones in DGI in mice.

MATERIALS AND METHODS

Mice. Caw:CF1 female mice (7 to 10 weeks old) were used in these experiments. They were fed commercial laboratory animal pellets and water ad libitum.

Neisseria gonorrhoeae. The N. gonorrhoeae strain used in these experiments was 57-120, which was provided by Y. Obara and S. Yamai, Kanagawa Prefectural Public Health Laboratory, Yokohama, Japan. Strain 57-120 was originally isolated from a human genital tract infection, preserved by the gelatin-disk method (36), and was resistant to mouse serum and sensitive to penicillin. Gonococci were grown on GC agar base supplemented with IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.), and cultures with greater than 95% of the colonies of T2 morphology were used.

For inoculation experiments, gonococci grown on GC agar plates were transferred to liquid medium, which was identical to the solid medium except that agar was omitted and 0.002% (wt/vol) cocarboxylase, 1% (wt/vol) glucose, and 50 mM NaHCO₃ were added. Gonococci in the liquid medium were cultured overnight at 37°C with continuous rotation as described previously (21).

Determination of the mean effective dose. Groups of 20 mice were injected subcutaneously each day with either 1 mg of hormones dissolved in 0.1 ml of sesame oil or sesame oil alone, beginning 3 days before infection. Mice were inoculated intraperitoneally with 0.2 ml of each dilution of gonococcal suspension in 10% gelatin (Difco Laboratories, Detroit, Mich.) and were monitored for disease for 1 week. The median (50%) effective dose (ED₅₀) was determined for two different parameters: (i) bacteremia at 24 h postinoculation and (ii) death within 72 h postinoculation (medium lethal dose). To determine bacteremia, 200 μl of blood was extracted from a capillary of the retro-orbital venous plexus of each mouse at 24 h postinoculation and then cultured on GC agar plus IsoVitaleX. To determine ED₅₀ by a probit transformation method (14), 10 mice at each dose level were used.

The kinetics of bacteremia after intraperitoneal inoculation of gonococci were determined in groups of 28 mice inoculated intraperitoneally with 10⁵ gonococci. Blood samples (200 μl) were obtained from the retro-orbital venous plexus at intervals of 1, 2, 3, 6, 12, 18, and 24 h after inoculation. At each time, four mice were randomly selected from each group, and quantitative cultures were performed after blood samples were diluted with saline.

Cell preparation. Mouse peritoneal polymorphonuclear leukocytes (PMN) were prepared by the method of Watt et al. (35). Briefly, mice were injected intraperitoneally with 2 ml of 0.2% (wt/vol) calcium caseinate and killed by cervical dislocation 3 h after injection.

The exudate cells were collected by washing the peritoneal cavity with 4 ml of RPMI 1640 (GIBCO Laboratories,
Grand Island, N.Y.) and centrifuged at 180 x 1000 g for 10 min. Contaminating erythrocytes were removed by suspending cells in NH₄Cl solution (0.168 M) at room temperature.

PMN were purified from caseinate-induced peritoneal cells by using the Percoll gradient as described by Watt et al. (35) and then harvested from the band corresponding to the density of 1.12 g/ml. Cells harvested from the band contained 98% or more PMN, irrespective of the hormones used for pretreatment of the mice.

The number of cells were counted with a hemocytometer by using the eosin dye exclusion test to estimate viability. Cell morphology was determined by Giemsa stain.

The viable PMN suspension harvested from the Percoll density gradient was adjusted to a concentration of 10⁶/ml in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (GIBCO).

Bactericidal and phagocytic tests. Phagocytosis was performed by incubating 1.0 ml of the caseinate-induced PMN suspension (10⁶ cells) with 1.0 ml of gonococci (10⁸ CFU) suspended in RPMI 1640 containing 10% heat-inactivated normal mouse serum in a screw-capped polystyrene tube (Falcon 2027 tube; 13 by 100 mm; Becton Dickinson Labware, Oxnard, Calif.) at 37°C under rotation. Tests were performed in quadruplicate for each PMN sample. After 30 min, the reaction mixture was centrifuged at 180 x g for 5 min and washed twice with RPMI 1640. Saponin (1 ml, 0.5%) was added to two pellet samples in test tubes randomly selected from each group, and the pellet samples were kept at 37°C for 10 min to disrupt leukocytes before dilution and enumeration; gonococci were not affected by this concentration of saponin. The number of viable bacteria recovered from these samples was designated CFU time 0. The same procedure was done with the rest of the samples 45 min after the reincubation started, and the CFU time 45 was measured. The bactericidal index of each PMN sample was expressed as (1 - CFU time 45/CFU time 0) x 100 (%).

The phagocytic activity of PMN was examined in the same reaction mixture as described above. After 30 min of incubation, the cells were recovered from the mixtures, concentrated by centrifugation at 180 x g for 5 min, and smeared on glass slides. Smears were fixed with methanol, stained with Giemsa solution, and examined microscopically for evidence of ingestion of gonococci by PMN. The results were expressed as the percentage of 200 PMN that contained at least one organism.

Superoxide generation and myeloperoxidase activity. Superoxide generation by PMN was determined by the spectrophotometric measurement of ferricytochrome c reduction (43).

The reaction mixture contained 10 µl of PMN suspension (10⁶ cells), 2 ml of Hanks balanced salt solution (HBSS) without phenol red containing 80 µM ferricytochrome c (Sigma Chemical Co., St. Louis, Mo.), 2 mM NaN₃, 10 mM sodium phosphate buffer (pH 7.4), and phorbol myristate acetate (4 µg/ml) in a Falcon test tube and was incubated at 37°C in a CO₂ chamber for 60 min. After incubation, 2 ml of cold HBSS was added to the reaction mixture. The mixture was centrifuged at 1,500 x g for 10 min at 2°C, and the supernatant was spectrophotometrically measured at 550 nm. All assays were carried out in triplicate, with controls containing superoxide dismutase (30 µg/ml; type I; Sigma) and PMN-free blanks. Release of superoxide was calculated from the difference in optical density at 550 nm (∆A₅₅₀) in the absence and the presence of superoxide dismutase, and the results were converted to nanomoles of reduced cytochrome c as follows: ∆E₅₅₀ = 2.1 x 10⁴ M⁻¹ cm⁻¹.

Myeloperoxidase (MPO) activity of PMN samples was measured in a spectrophotometer at 460 nm by the method of Alexander and Meakins (1). The PMN suspension was centrifuged, and 2 ml of acetate buffer (pH 3.7) was added to the cell pellet (5 x 10⁷ cells per ml). The PMN were disrupted by sonication and centrifuged at 20,000 x g for 30 min. The reaction mixture consisted of 0.25 ml of the supernatant, 0.3 ml of H₂O₂, 0.05 ml of 20 mM O-dianisidine (Sigma) in methanol, 0.3 ml of 0.1 M phosphate buffer (pH 6.0), and 2.1 ml of distilled water. The activity was expressed as a unit of MPO activity, which was defined as that activity causing an increase in absorbancy of 10⁻³/min.

Peritoneal cell responses. To determine the cellular responses in the peritoneal cavity, peritoneal fluid was withdrawn with 3 ml of phenol red-free HBSS from four mice of each group 30 min before inoculation with 10⁶ Formalin-killed gonococci. After 3 and 12 h, peritoneal exudate was collected in the same way from different batches of mice in each group. The fluid was concentrated by centrifugation at 180 x g for 10 min, and the pelleted cells were suspended in 1 ml of HBSS for total and differential cell analyses.

Susceptibility of gonococci to killing by mouse serum. Groups of 10 mice receiving hormone injections were used to prepare the pooled serum, and the bactericidal test was done by the method described by McCutchan et al. (26) with a slight modification. Briefly, equal volumes of mouse serum and gonococcal suspension (10⁵ CFU suspended in RPMI 1640) were mixed and incubated for 1 h at 37°C in 5% CO₂. After serial 10-fold dilutions of each mixture were made, duplicate 0.1-ml portions of diluted samples were spread to dryness on plates composed of clear typing medium (34) supplemented with 2% (wt/vol) agar, 0.002% (wt/vol) cocarboxylase, and 1% (wt/vol) glucose, and culture was carried out overnight at 37°C in 5% CO₂. Bactericidal activity of each serum was expressed as the log₁₀ of the number of organisms killed after the 1-h incubation.

Hormones. Estrogen (17β-estradiol) and progesterone were obtained from Sigma Chemical Co. and dissolved in sesam oil before use.

Statistical analysis. Results were expressed as means ± standard error and compared by Student’s t test. Differences were considered significant when the probability (P) was <0.01.

RESULTS

Effect of reproductive hormones on DGI. Before the experiments were begun, the effect of hormone administration was determined by vaginal smears. The estrogen effect was identified by vaginal smears consisting of only epithelial cells, and the progesterone effect was identified by smears containing PMN and cornified cells. Two different criteria for gonococcal infection, i.e., positive blood culture at 24 h postinoculation and death within 72 h postinoculation, were used to determine ED₅₀ values. Results are summarized in Table 1. The ED₅₀ for bacteremia in mice of the estradiol-treated group, 4.8 logs lower than that in mice of the control group (10⁸.⁶, P < 0.001). When mice were treated with progesterone, resistance was enhanced and positive results were not obtained by either of the two criteria. The ED₅₀ for death was 10⁸.⁴ in mice of the estradiol-treated group, and the difference in ED₅₀ between bacteremia and death was 1.6 logs. While the ED₅₀ for bacteremia in mice of both control groups was 10⁷.⁶, mice could not survive the challenge with 10⁷.⁹. The difference in ED₅₀ for death between the estradiol-treated and control groups was 3.9 logs. These results indicate that estradiol treatment can enhance the susceptibil-
entity of mice to DGI by at least 10^4-fold compared with controls.

**Kinetics of bacteremia in treated mice.** To study the course of bacteremia in hormone-treated mice, mice were injected intraperitoneally with 10^6 gonococci. In mice treated with estradiol, bacteremia progressively developed within 12 h postinoculation, and mice died within the next 6 h (Fig. 1). Infection with 10^6 gonococci produced transient bacteremia in two control groups which terminated within 18 h postinoculation, whereas bacteremia observed in mice treated with progesterone was completely cleared within 3 h postinoculation.

**Effect of reproductive hormones on PMN activities.** To determine the effect of reproductive hormones on the activity of PMN, peritoneal PMN were collected from mice treated with hormones. As shown in Table 2, differences in the phagocytic activity of PMN between groups were not observed as determined by the percentage of phagocytosis. However, intracellular killing by PMN was significantly decreased in mice treated with estradiol. In contrast, phagocytosis by PMN derived from mice of other groups was strongly bactericidal, and differences between bactericidal activities were not observed.

To clarify the reason why bactericidal activity of PMN collected from the estradiol-treated mice is low, superoxide production and MPO levels in PMN were measured. As shown in Table 3, the levels of superoxide generation were equivalent among the four groups, but the MPO level of PMN collected from the estradiol-treated mice was significantly lower (P < 0.01).

**Kinetics of peritoneal cell responses.** After mice subcutaneously received sex hormones for three consecutive days, the mean number of peritoneal macrophages was not significantly different between groups (Fig. 2). However, PMN counts in the estradiol-treated mice slightly decreased compared with those in control and progesterone-treated mice. The mean total number of peritoneal leukocytes obtained from control mice was 7.25 x 10^6 ± 0.14 x 10^6, and less than 2% of these cells were PMN (macrophages, 54%; lymphocytes, 40%; others 4%) as judged by morphological characteristics. In hormone-treated mice, the number of peritoneal leukocytes was 2.5 x 10^6 ± 0.23 x 10^6 (macrophages, 58%; lymphocytes, 40%; PMN, 6.6%; others, 1.4%) in estradiol-treated mice and 4.28 x 10^6 ± 0.13 x 10^6 (macrophages, 37%; lymphocytes, 48%; PMN, 5%; others, 10%) in progesterone-treated mice.

At 3 h after an intraperitoneal inoculation of 10^6 Formalin-killed gonococci, the mean total number of leukocytes slightly increased in both control mice (9.58 x 10^6 ± 0.24 x 10^6) and progesterone-treated mice (9.32 x 10^6 ± 0.13 x 10^6). This was due to an increase in the number of PMN in

### Table 1. Effect of reproductive hormones on the infectivity of intraperitoneally injected *N. gonorrhoeae* in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log_{10} of ED_{50}</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>8.6</td>
<td>9.3</td>
</tr>
<tr>
<td>Estradiol</td>
<td>3.8\textsuperscript{b}</td>
<td>5.4\textsuperscript{b}</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&gt;9.5\textsuperscript{c}</td>
<td>&gt;9.5\textsuperscript{c}</td>
</tr>
<tr>
<td>Sesame oil</td>
<td>8.6</td>
<td>9.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Ten mice were used at each dose level to determine ED_{50}. Bacteremia, positive blood culture at 24 h postinoculation; Death, death occurring within 72 h postinoculation.

\textsuperscript{b} The decreases in ED_{50} for the estradiol-treated group were statistically significant (P < 0.001) compared with the untreated group.

\textsuperscript{c} The progesterone-treated group (described as >9.5) had no deaths or positive cultures.

### Table 2. Effect of reproductive hormones on phagocytic and bactericidal activities of mouse peritoneal PMN

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PMN activity (mean ± SE)</th>
<th>Bactericidal index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>39 ± 3</td>
<td>25 ± 4\textsuperscript{b}</td>
</tr>
<tr>
<td>Progesterone</td>
<td>40 ± 5</td>
<td>86 ± 5</td>
</tr>
<tr>
<td>Sesame oil</td>
<td>42 ± 4</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>Untreated</td>
<td>40 ± 3</td>
<td>85 ± 6</td>
</tr>
</tbody>
</table>

\textsuperscript{b} Both tests were performed in quadruplicate, and data were obtained from three different experiments. Phagocytic activity was determined as the mean percentage of 200 PMN that contained at least one organism. The bactericidal index (percent killing) was determined as follows: (1 – CFU time 45/CFU time 0) x 100 (%) ± SE.

\textsuperscript{c} Statistically significant (P < 0.01) compared with the untreated group.

### Table 3. Effect of reproductive hormones on superoxide generation and MPO levels of mouse peritoneal PMN

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Superoxide generation (nmol/10^6 PMN per 90 min)</th>
<th>MPO activity (U/10^6 PMN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>20.4 ± 2.5</td>
<td>4.3 ± 1.2\textsuperscript{c}</td>
</tr>
<tr>
<td>Progesterone</td>
<td>24.2 ± 1.8</td>
<td>17.1 ± 1.5</td>
</tr>
<tr>
<td>Sesame oil</td>
<td>21.2 ± 2.4</td>
<td>15.8 ± 1.2</td>
</tr>
<tr>
<td>Untreated</td>
<td>22.4 ± 1.5</td>
<td>16.4 ± 2.1</td>
</tr>
</tbody>
</table>

\textsuperscript{c} Tests were performed in triplicate, and data were obtained from three different experiments. Results are expressed as nanomoles of cytochrome c reduced per 10^6 PMN for 90 min ± SE. There was no statistically significant difference between groups.

\textsuperscript{c} Tests were performed in duplicate, and data were obtained from three different experiments. Results are expressed as units of MPO activity ± SE, with a unit defined as the activity causing an increase in absorbancy of 10^-5/min.

\textsuperscript{c} Statistically significant (P < 0.01) compared with the untreated group.
activity against gonococci. The results (Table 4) demonstrated that only the progesterone-treated mouse serum could decrease the viable gonococci by 2.4 logs. None of the serum samples obtained from control and estradiol-treated mice killed gonococci to a significant extent.

**DISCUSSION**

Corbeil et al. (12) reported that the mucin-hemoglobin mixture could enhance gonococcal infection in mice. The mucin-hemoglobin mixture did not function directly as a growth-promoting nutrient substance but seemed to affect local peritoneal defenses by destroying peritoneal exudate cells (predominantly PMN), allowing gonococci to persist. In our previous study (21), we found that female mice at the late proestrus or early estrous stage, periods when estrogen levels are highest, are the most susceptible to genital infection with *N. gonorrhoeae*. Since gonococcal dissemination usually occurs at menstruation and during pregnancy (16, 17), hormonal imbalance as well as menstruum (containing mucin and hemoglobin) may be responsible for gonococcal progression from local infection to bacteremia. It is generally accepted that progesterone antagonizes certain effects of estrogen (8, 9, 23), especially in reducing the level of cytoplasmic estrogen receptor (7, 18, 19, 24, 28). This is closely related to the report (33) that estrogen can stimulate the bacterial adherence to HeLa cells. Thus, it might be suggested that the resistance of diestrous female mice against gonococcal genital infection can be accounted for by the anti-estrogenic effect of progesterone, because levels of progesterone in serum are highest at this stage. However, when the peritoneal route is used for challenge, it appears that the effect of estrogen on bacterial cell attachment does not account for the enhanced infection because the interaction between gonococci and phagocytic cells in the peritoneal cavity of the estradiol-treated mice was similar to that in other groups as demonstrated by phagocytic activity. However, intracellular killing by PMN was significantly impaired by estradiol treatment. Release of superoxide anion by PMN was not impaired by hormone injection, but MPO activity was affected by estradiol.

Klebanoff (22) reported that the MPO-H₂O₂-halide bacterial system was inhibited by estradiol when the H₂O₂ and halide concentrations were low. Although the actual concentrations of H₂O₂ and halide in mouse peritoneal PMN were not determined in this study, the decreased level of MPO in the estradiol-treated PMN can partly account for the low level of intracellular gonococcal killing by PMN. In conjunction with this, the anti-inflammatory effects of estrogens have been demonstrated (6).

Analysis of peritoneal cells in mice treated with hormones demonstrated that PMN responses after inoculation with gonococci were obviously depressed in mice treated with estradiol. The depressed PMN responses in the peritoneal cavities of estradiol-treated mice were also observed when mice were inoculated intraperitoneally with heat-killed *Staphylococcus aureus* (data not shown), so the failure of PMN to infiltrate intraperitoneally in the estradiol-treated mice is not specific to gonococcal inoculation. Furthermore, the number of PMN in the peritoneal cavity of mice treated with estradiol was lower by at least 0.9 log compared with other groups. This may be due to the anti-inflammatory effect of estrogen, and it can be speculated that PMN chemotaxis may be affected by estrogen.

It has been reported (32) that estrogen can stimulate the uptake of sheep erythrocytes by the reticuloendothelial system. In this study, however, bacteremia following intra-

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**TABLE 4. Bactericidal activity of hormone-treated mouse serum against gonococci**

<table>
<thead>
<tr>
<th>Serum*</th>
<th>Avg log₁₀ kill†</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM</td>
<td>0.8</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.7</td>
</tr>
<tr>
<td>Estradiol</td>
<td>0.6</td>
</tr>
<tr>
<td>Progesterone</td>
<td>2.4*</td>
</tr>
<tr>
<td>Human</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* Serum samples tested in this study were pooled normal mouse serum (NM), 10% gelatin-injected mouse serum (Gelatin), estradiol-injected mouse serum (Estradiol), progesterone-injected mouse serum (Progesterone), and normal human serum.

† Average log₁₀ kill was obtained from four different tests and expressed as the log of the number of killed organisms after 1 h of incubation.

* Statistically significant (P < 0.01) compared with normal mouse serum.
peritoneal inoculation of gonococci became much more severe in estradiol-treated mice than in other groups. One possible explanation is that the uptake of gonococci by the reticuloendothelial system could be enhanced in the estradiol-treated mice but that the intracellular killing after ingestion might be impaired.

Superoxide anion is a precursor of H$_2$O$_2$, HOCl, OH$, and other more active oxygen intermediates which have microbicidal activity, and its production by PMN collected from the estradiol-treated mice was similar to the results obtained with PMN from other groups. It is not clear why PMN of the estradiol-treated mice cannot kill gonococci to the same extent as do the PMN of other groups. It remains to be determined whether gonococci are more susceptible to the MPO-H$_2$O$_2$-halide bacterial system than to oxygen intermediates.

The gonococcal strain used in this study is resistant to fresh mouse serum and highly sensitive to fresh human serum. The estradiol-treated mouse serum had less bactericidal activity against gonococci (log$_{10}$ kill, 0.6), but progesterone treatment slightly enhanced the antigonococcal activity of mouse serum (log$_{10}$ kill, 2.4). The enhanced antigonococcal activity of serum in the progesterone-treated mouse can account for shortening the duration of bacteremia in mice of this group. The degree of antigonococcal activity in each serum was not affected by heating the serum at 56°C for 30 min (data not shown).

The actual concentrations of each hormone in serum used here occur naturally, and the hormone treatment used in this study resulted in the same hormonal levels observed in pregnant mice (estradiol, 20 ng/ml; progesterone, 170 ng/ml; as determined by radioimmunoassay).

Although treatment with estradiol induced the enhanced host susceptibility to gonococci by impairing the PMN response in mice, there seem to be other, more complicated biological changes, especially with regard to liver function because estrogen is usually inactivated and the reticuloendothelial system is active in this organ. Since the liver is responsible for lipid metabolism and gonococci are very sensitive to some fatty acids, the liver function which might be affected by estradiol treatment is believed to play an important role in controlling the multiplication of gonococci.

Thus, a study is in progress to clarify both the histological and biochemical changes in the livers of mice treated with hormones and also mechanisms under which PMN function is affected by estradiol.

ACKNOWLEDGMENTS

We thank S. Yamai and Y. Obara for providing the strain of N. gonorrhoeae used in this work.

LITERATURE CITED


