Oestrogen binding by and effect of oestrogen on trichomonads and bacteria

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Summary. It has been shown previously that *Trichomonas vaginalis*, a common protozoan pathogen of the female genito-urinary tract, has binding sites for oestrogen and that its growth, adherence and chemotaxis are altered after exposure to physiological concentrations of oestrogens. Two other species of trichomonad showed no physiological response to oestrogens but did have high affinity oestrogen binding with low binding capacity. To determine whether oestrogen binding sites occur in other pathogenic micro-organisms, these studies with eukaryotic pathogens were extended to prokaryotic bacteria. Numerous bacteria previously considered to be oestrogen responsive, other species of the same genera, and control bacteria (not considered oestrogen responsive) were examined. High affinity protein-containing oestrogen binding sites were demonstrated in several bacterial genera. Direct oestrogenic effects on micro-organisms, as well as the traditional oestrogenic effects on host cells, may explain why certain infections are more common or more severe after exposure to oestrogen.

Introduction

The hormonal milieu has been shown to alter mammalian host susceptibility to numerous infections.¹⁻³ Corticosteroids have been studied most in this regard and are well known for changing the immune response of the host. However, oestrogens also alter susceptibility to infection at various stages of the menstrual cycle or when taken as supplements, e.g., as oral contraceptives.²⁻⁴ Reported mechanisms of these oestrogen actions include: (1) oestrogen-mediated alterations of the immune response of the host; (2) alterations in other host defense factors such as the production of cervical mucus in the genitourinary tract; and (3) oestrogen-mediated alterations of mammalian cell structure. Scattered reports of the demonstration of oestrogen- and other hormone-binding sites in, and effects of oestrogens on, *Trichomonas vaginalis* and several other eukaryotic micro-organisms lend support to the hypothesis that oestrogens may directly alter the virulence of micro-organisms.⁵⁻⁹ However, reported binding site dissociation constants are often far greater than normal human serum levels of oestrogen.

The situation with (prokaryotic) bacteria is much more confusing. It is well established that infections caused by many bacterial pathogens, particularly sexually transmitted pathogens such as *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, are more common and more severe at certain stages of the menstrual cycle in women, or in women who are taking supplemental oestrogens.¹⁰⁻¹² In the last decade active research on the direct hormonal responsiveness of bacteria has yielded interesting findings, e.g., various recent reports have suggested that virulence factors of certain pathogenic bacteria can be altered after exposure to physiological concentrations of oestrogens.¹³⁻¹⁵ However, in most of these studies and other references cited in table III,¹⁶⁻¹⁸ the system employed does not exclude the possibility that oestrogenic effects on mammalian receptor cells are, at least partially, responsible for the differences observed. More direct evidence of bacterial response to hormones has been published recently. This includes the synthesis by *Clostridium* spp. of enzymes that can convert cortisol to androgen reported in 1984.¹⁹ A saturable binding site for the hormone thyrotropin has been demonstrated in *Yersinia enterocolitica*²⁰ and an oestrogen-binding protein in *Pseudomonas aeruginosa*.²¹

We have attempted to determine whether oestrogen-binding sites and oestrogen responsiveness are common in eukaryotic and prokaryotic pathogenic
micro-organisms, to support further the hypothesis that oestrogens may directly modulate the virulence of such pathogens.

Materials and methods

Trichomonads

*T. vaginalis* isolates were cultivated from vaginal specimens in which direct microscopy of saline wet mounts showed viable organisms. The specimens were inoculated into borosilicate screw-capped glass tubes (8 × 100 mm) containing 6 mL of Linstead's DL8 defined medium, pH 7.2, supplemented with human serum 8%, gentamicin 40 mg/L and amphotericin B 2.5 mg/L.22 Cultures were maintained in DL8 medium with heat-inactivated equine serum 4% (instead of human serum) at 37°C in air and were subcultured every 3-4 days (after reaching the stationary phase). Antibiotics were omitted from the medium after several passages and this was the growth medium used for all trichomonads in experiments. Strains ATCC 30227 and 30230 of the gastrointestinal tract pathogen of birds, *T. gallinae* (American Type Culture Collection, Rockville, MD, USA) were maintained in CTLM medium (ATCC media formulations no. 745), washed and resuspended in growth medium for all assays. Isolates of *Trichomonas foetus* (a bovine genitourinary pathogen) were obtained from clinical specimens from cows; one isolate (D1-3) was cloned three times to obtain a homogenous population. These isolates were maintained in CTLM medium and grown for assays in DL8 medium.

Bacteria

Reference strains and clinical isolates of the following bacterial species were tested for the presence of oestrogen-binding sites: *Escherichia coli* (ATCC 25922 and 2 clinical isolates); *E. blattae* ATCC 29907; *E. hermannii* ATCC 33650; *Klebsiella pneumoniae* (2); *Proteus mirabilis* (2); *P. vulgaris* (2); *Pseudomonas aeruginosaa* (2); *Staphylococcus aureus* (ATCC 29213 + 2); *S. intermedius* (1); *S. epidermidis* (2); *Streptococcus pyogenes* (3); *Str. agalactiae* (2); *Str. pneumoniae* (2); α-haemolytic streptococcus (1); *Enterococcus faecalis* (2); *Neisseria gonorrhoeae* (2); *N. meningitidis* (2).

Oestrogen

17-β-oestradiol (Sigma) was dissolved initially in absolute ethanol, then diluted in phosphate-buffered saline (PBS; 0.15 M sodium chloride, 0.15 M sodium phosphate; pH 7.3) to provide stock solutions of oestrogen at concentrations 100 times the test concentrations and containing ethanol <0.1% v/v. Baseline trichomonad medium contained <0.1% of the lowest final oestrogen concentration in test media with supplemental oestrogen (1 ng/mL, 3.7 × 10⁻⁹ M). This lowest test concentration is about 10 times the maximum normal human plasma concentration.

Growth assay

Cultures of trichomonads were centrifuged at 250 g for 5 min and resuspended in warmed medium. After vortex mixing, an inoculum of c. 2.5 × 10⁵ organisms was added to each tube containing 6 mL of growth medium plus oestrogen or PBS (control). These tubes were incubated for 3 days, then centrifuged, and the supernate was removed; the harvested trichomonads were suspended in 1-0 mL of warmed PBS and the number of organisms was counted in a haemocytometer.

Radiolabelling

Exponential-phase trichomonad isolates in growth medium were radiolabelled with ³H-thymidine (specific activity 78.3 Ci/mmol, 2.9 TBq/mmol; DuPont, NEN Research Products, Boston, MA, USA) 5 μCi (185 kBq)/mL for 18 h at 37°C. Organisms were washed thrice in PBS by centrifugation before further use in assays.

Chemotaxis assay

Radiolabelled organisms were washed in PBS and resuspended in growth medium to a concentration of 1 × 10⁵ trichomonads/mL (verified by haemocytometry) and 0.5-ml samples of the suspension were placed in the wells of a 24-well Transwell plate (Costar, Cambridge, MA, USA). A polycarbonate membrane filter (0.45-μm pore size) insert (small enough to trap organisms, but large enough for free passage of medium and chemotactic agents) was placed on top of the suspension, and 0.5 mL of medium was placed on top of the membrane. Concentrated (stock solution; 100 × final concentration) supplemental oestrogen, or an equal volume of PBS (control) was added to the top of the well to yield the final test concentration. Migrating trichomonads were trapped in the filter during incubation at 37°C for 45 min. The membrane insert was then removed and inverted, and excess fluid was removed by blotting with filter paper. The membranes were removed from the inserts and dissolved in 0.5 mL of 0.5 M Protosol (DuPont, NEN Research Products); scintillation fluid was added, and vials were placed in a liquid scintillation counter. Duplicate experiments were performed with known numbers of labelled trichomonads counted in the liquid scintillation counter to allow conversion of all cpm measurements to numbers of trichomonads. This assay was described in detail elsewhere.23

Adherence assay

McCoy cells (mouse fibroblasts) were grown in Eagle's Minimum Essential Medium with Earle's salts (GIBCO Laboratories, Grand Island, NY, USA), pH 7.2, plus fetal bovine serum (HyClone Laboratories, Inc. Logan, UT, USA) 10% v/v. These cells were grown to confluence (about 2 × 10⁵ cells/well) in 24-well (16-mm diameter) Costar culture plates in an atmosphere of air + CO₂ 5% at 37°C. After removal of the medium from confluent
monolayers, 1-0 ml of medium consisting of two parts mammalian cell culture medium and one part trichomonad growth medium, containing $2 \times 10^6$ radiolabelled trichomonads previously incubated at 37°C for 1 h with oestrogen or PBS, was added. After incubation at 37°C in air + CO$_2$ 5% for 45 min, non-adherent trichomonads were removed by washing three times in warm PBS. The contents of each well (McCoy cells and adherent trichomonads) were then digested with 0-5 ml of 0-1 M NaOH for 30 min on a shaking apparatus. The digest was neutralised to pH 7 with HCl and transferred to vials to which scintillation fluid was then added, and the radioactivity was measured in a liquid scintillation counter; the cpm were converted to the number of trichomonads as described previously.

**Determination of oestrogen binding sites**

Trichomonads were cultivated in 100 ml of growth medium to the stationary phase (c. 3 days), and washed three times in PBS and resuspended in 5 ml of 10 mM Tris, 1-5 mM EDTA, 250 mM sucrose, 12 mM monothioglycerol, and 10 mM sodium molybdate buffer, pH 7-4, at 4°C. They were surrounded by ice and disrupted with two 5-s bursts of sonication with the lowest amount of energy which disrupted the cells as verified microscopically. In an alternative procedure organisms were lysed by freezing and thawing three times in acetone-dry ice. Large cellular fragments were removed by centrifugation at 1000 g for 10 min at 4°C and the supernate was then centrifuged at 100 000 g for 2 h. At least six concentrations of oestradiol were gently shaken and incubated at 4°C for 3 h. Bound oestradiol was separated from free oestradiol by two centrifugation filtrations through 10 000 mol.-wt cut-off low-protein-binding cellulosic membrane ultrafiltration units (Millipore, Bedford, MA, USA). Radioactivity of the retentates was counted in a liquid scintillation counter. The calculated counting efficiency of $^3$H-oestradiol was 32% and background counts averaging 30-40 cpm were deducted from all calculations. Standard techniques were used to determine affinity and dissociation constants (K$_a$, K$_d$), and binding capacities. Verification of the presence of protein in the binding sites was by incubation of the cytosol for 30 min with Trypsin (Sigma) 100 µg/ml or PBS at 37°C, cooling, and then performing the binding assay.

Oestrogen-binding sites of fresh clinical and stock isolates of several bacterial species were assayed in a similar manner, but with the bacteria grown overnight in 50 ml of Trypticase Soy Broth (BBL Microbiology Systems, Cockeysville, MD, USA). Cultures were lysed by freezing and thawing three times and cytosol supernates were prepared by centrifugation at 30 000 g for 20 min and then 100 000 g for 2 h. At least six concentrations of radiolabelled oestradiol, up to $8 \times 10^{-8}$ M were added.

**Statistical methods**

All growth, chemotaxis and adherence experiments were performed at least four times and oestrogen-binding experiments at least three times. Results were analysed with two-tail Student's $t$ tests. Data are expressed as mean and standard deviation (SD).

**Results**

**Effect of oestrogen on *T. vaginalis***

Previously reported experiments with fewer isolates were repeated with nine *T. vaginalis* isolates and non-estradiol oestradiol were gently shaken and incubated at 4°C for 3 h. Bound oestradiol was separated from free oestradiol by two centrifugation filtrations through 10 000 mol.-wt cut-off low-protein-binding cellulosic membrane ultrafiltration units (Millipore, Bedford, MA, USA). The mixtures of cytosol and oestradiol were gently shaken and incubated at 4°C for 3 h. Bound oestradiol was separated from free oestradiol by two centrifugation filtrations through 10 000 mol.-wt cut-off low-protein-binding cellulosic membrane ultrafiltration units (Millipore, Bedford, MA, USA). Radioactivity of the retentates was counted in a liquid scintillation counter. The calculated counting efficiency of $^3$H-oestradiol was 32% and background counts averaging 30-40 cpm were deducted from all calculations. Standard techniques were used to determine affinity and dissociation constants (K$_a$, K$_d$), and binding capacities. Verification of the presence of protein in the binding sites was by incubation of the cytosol for 30 min with Trypsin (Sigma) 100 µg/ml or PBS at 37°C, cooling, and then performing the binding assay.

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**Results**

**Effect of oestrogen on *T. vaginalis***

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**Table I. Effect of oestriadiol on the growth, adherence and chemotaxis of trichomonads other than *T. vaginalis***

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean (SD) number ($10^3$) of trichomonads in tests for growth at 72h</th>
<th>adherence to McCoy cells after 45min</th>
<th>chemotaxis*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Oestradiol 1 ng/ml</td>
<td>Control</td>
</tr>
<tr>
<td><em>T. foetus 1</em></td>
<td>1280 (153)</td>
<td>1367 (361)</td>
<td>347 (94)</td>
</tr>
<tr>
<td><em>T. foetus D1-3</em></td>
<td>1146 (201)</td>
<td>1194 (158)</td>
<td>396 (92)</td>
</tr>
<tr>
<td><em>T. gallinae ATCC 30227</em></td>
<td>769 (478)</td>
<td>761 (476)</td>
<td>307 (12)</td>
</tr>
<tr>
<td><em>T. gallinae ATCC 30230</em></td>
<td>893 (585)</td>
<td>797 (487)</td>
<td>320 (39)</td>
</tr>
</tbody>
</table>

*Trichomonads on filter after incubation for 45 min with organisms below filter and oestrone above.
and the effects of oestrogen observed with all isolates—significantly decreased growth, decreased adherence to mammalian cells and chemorepulsion after exposure to oestrogen—were similar to those already reported (data not shown).8,23

Effect of oestrogen responsiveness on other trichomonads

Two isolates each of T. foetus and T. gallinae were unaffected by oestrogens in the same assay systems (table I). In none of the isolates was there altered growth, altered adherence to McCoy cells or altered chemotaxis after exposure to oestradiol. However, all strains did display chemotaxis, manifest by chemo-attraction to nutrient medium from PBS (average 119% of baseline) without change in chemokinesis, as described previously for T. vaginalis isolates.8

Oestrogen-binding sites

The presence of oestrogen-binding sites was determined with stock cultures containing about (5–6) × 10⁶ trichomonads/ml. Results were the same when trichomonads were lysed by sonication or by freezing and thawing (data not shown). All trichomonads displayed saturable specific oestrogen binding. The protein nature of these binding sites was confirmed by repeating oestrogen-binding studies with cytosol preparations incubated with trypsin. Mean specific binding after exposure to trypsin was 8-2 (SD 11-0)% of control binding for five trichomonads tested. The possibility that contamination with binding sites from residual medium in the cytosol preparation could have altered the results was refuted by radiotracer studies in which cytosol preparations were found to contain <0-00002% v/v of medium, yet either growth medium or CTLM medium 0-0001% v/v in cytosol buffer did not bind ³H-oestradiol in 8 × 10⁻¹⁰ M solution.

The oestrogen binding affinity (Ka, Kd) was similar for all isolates; however, significant differences in oestrogen-binding capacity were noted between T. vaginalis and the other trichomonads. Representative data are given in table II. T. vaginalis displayed significantly greater (p < 0-05) total oestrogen binding capacity than other trichomonads which were not oestrogen responsive (table II).

Oestrogen binding sites in bacteria

High affinity, saturable oestrogen-binding sites were found in fresh isolates and stock cultures of several species of pathogenic bacteria (table III). More than 95% of these sites were destroyed by incubation with trypsin. Binding sites were found in several species of the same genera (table III). Binding sites were not identified in other genera, including some traditionally thought to be oestrogen responsive such as N. gonorrhoeae. None of the test strains of K. pneumoniae, N. gonorrhoeae, N. meningitidis, P. mirabilis, P. vulgaris, S. epidermidis, Str. pneumoniae and Ent. faecalis displayed any specific oestrogen binding with up to 8 × 10⁻⁸ M ³H-oestradiol.

Discussion

These data show the presence of protein-containing oestrogen-binding sites of similar high affinity in different trichomonads, including those which are not primarily pathogens of the female genital tract. T. vaginalis, a common genitourinary pathogen of women, responds to oestrogens and has significantly higher oestrogen-binding capacity than the non-human trichomonads which do not respond by physiological changes. Several species of bacteria were also shown to have high affinity protein-containing oestrogen-binding sites with low binding capacities; however, unlike the trichomonads, other species of the same genera sometimes did not. Certain bacteria thought to be oestrogen responsive, e.g., N. gonorrhoeae, had no specific oestrogen-binding sites.

The effects of oestrogens on potentially pathogenic micro-organisms may be quite different from the effects on the host. Thus, oestrogens seem to decrease the infectivity of T. vaginalis in vitro,8,23 but the few reports of animal studies available suggest that oestrogens increase the infectivity of T. vaginalis in vivo.25,26 Interaction between the
potentially divergent effects of hormones on microorganisms and on the host may determine alterations in infectivity. The presence of high affinity oestrogen-binding sites in many prokaryotic and eukaryotic micro-organisms adds strength to the hypothesis that oestrogens may directly alter their virulence. Further studies of oestrogen binding and physiological responsiveness of other micro-organisms may help to determine the occurrence and clinical significance of direct oestrogen effects on micro-organisms.

This work was supported, in part, by Michigan State University. Dr Bob BonDurant at the University of California, Davis kindly supplied the cloned \textit{T. foetus} isolate. We thank Diane Bannerman for expert secretarial assistance.

REFERENCES

15. Sobel JD, Kaye D. Enhancement of \textit{Escherichia coli}

Table III. Oestrogen binding activity of selected bacteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oestrogen responsiveness (selected references)</th>
<th>Oestrogen binding activity</th>
<th>Dissociation constant (Kd)(^*)</th>
<th>Binding capacity(^*)(\uparrow)</th>
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<tbody>
<tr>
<td>\textit{E. blattae} ATCC 29907</td>
<td>...</td>
<td>Yes</td>
<td>13.5 (3.8)</td>
<td>0.4 (0.2)</td>
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<tr>
<td>\textit{E. coli} ATCC 25922</td>
<td>(15–17)</td>
<td>Yes</td>
<td>3.4 (1.9)</td>
<td>5.8 (2.9)</td>
</tr>
<tr>
<td>1</td>
<td>Yes</td>
<td>2.6 (1.1)</td>
<td>4.7 (2.8)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>4.2 (1.4)</td>
<td>1.9 (0.7)</td>
<td></td>
</tr>
<tr>
<td>\textit{E. hermanii} (ATCC 33650)</td>
<td>...</td>
<td>Yes</td>
<td>8.7 (2.4)</td>
<td>1.6 (0.4)</td>
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<tr>
<td>\textit{P. aeruginosa} 1</td>
<td>...</td>
<td>Yes</td>
<td>21.5 (5.6)</td>
<td>2.6 (0.9)</td>
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<tr>
<td>2</td>
<td>Yes</td>
<td>16.8 (4.1)</td>
<td>1.3 (0.5)</td>
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<tr>
<td>\textit{S. aureus} ATCC 29213</td>
<td>(18)</td>
<td>Yes</td>
<td>1.6 (0.7)</td>
<td>3.6 (2.1)</td>
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<tr>
<td>1</td>
<td>Yes</td>
<td>5.2 (1.9)</td>
<td>2.7 (1.6)</td>
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<tr>
<td>2</td>
<td>Yes</td>
<td>11.1 (3.0)</td>
<td>1.9 (0.8)</td>
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<tr>
<td>\textit{S. intermedius} 1</td>
<td>...</td>
<td>Yes</td>
<td>9.2 (3.1)</td>
<td>0.6 (0.3)</td>
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<tr>
<td>\textit{Str. agalactiae} (group B) 1</td>
<td>(13)</td>
<td>No</td>
<td>...</td>
<td>0</td>
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<td>2</td>
<td>No</td>
<td>...</td>
<td>0</td>
<td></td>
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<tr>
<td>\textit{Str. pyogenes} 1</td>
<td>...</td>
<td>Yes</td>
<td>16.2 (3.3)</td>
<td>1.1 (0.9)</td>
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<td>3</td>
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<td>...</td>
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<td></td>
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<tr>
<td>z-haemolytic streptococcus 1</td>
<td>...</td>
<td>Yes</td>
<td>12.5 (2.9)</td>
<td>1.2 (0.4)</td>
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</table>

\(^*\)See footnote to table II.
\(^\uparrow\)Preparation contained cytosol protein 700–1000 mg/L.


