A Proposed Role for the Lutropin Receptor in Contact-Inducible Gonococcal Invasion of Hec1B Cells

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We previously reported the existence of a contact-inducible, enhanced invasion phenotype in the obligate human pathogen *Neisseria gonorrhoeae*. Our present studies showed that the ability of glutaraldehyde-fixed eucaryotic cells to convert gonococci (GC) to this invasive phenotype (Inv⁺) is limited to cells derived from reproductive tissues. We present evidence that GC recognize the lutropin receptor (LHR), which recognizes both luteinizing hormone and human chorionic gonadotropin (hCG), as the tissue-specific environmental signal that induces the conversion of GC to the Inv⁺ phenotype. By competitive binding studies, we showed that Inv⁺ GC bind to Hec1B cells, a human endometrial cell line, by a unique adhesin not present on noninduced GC and that this Inv⁺ GC-specific binding is completely blocked by the addition of hCG. We demonstrated that limiting the access of GC to LHR decreases the ability of the host cell to both convert GC to the Inv⁺ phenotype and serve as a target for Inv⁺ GC invasion. We propose a model of GC invasion of Hec1B cells in which the LHR plays a dual role both as an induction signal and as part of the internalization mechanism. This utilization of LHR could account for both the preponderance of complicated GC disease in women and the observed correlation of the disease with the onset of menses.

Neisseria gonorrhoeae, the causative agent of gonorrhea, is a readily treated sexually transmitted disease. Yet gonococcal infections remain the contagious disease most commonly reported to the Centers for Disease Control and Prevention (1). Gonorrhea can progress into salpingitis, a serious infection of the fallopian tubes. This type of complicated gonococcus (GC) infection often leads to pelvic inflammatory disease (PID), with a subsequent increased risk of infertility, ectopic pregnancy, and recurrent episodes of PID. In many cases, the initial PID episode damages the fallopian tube in such a way that it is subsequently susceptible to reinfection by normal bacterial flora of the lower reproductive tract (27). This enhanced susceptibility to recurrent PID episodes means that efforts to limit initial infections by highly virulent organisms have a greatly magnified effect on total numbers of future PID cases. In up to 50% of women with gonorrhea, the easily treated infection of the lower reproductive tract is asymptomatic, and it is only when the infection progresses to complicated disease that treatment is sought (4). This asymptomatic nature of uncomplicated gonorrhea is the primary reason why GC PID remains a major women's health issue. Thus, understanding the mechanism by which the often asymptomatic GC infection in women invades the cells of the fallopian tubes is crucial for preventing GC PID.

For many pathogens, host cell invasion occurs by the triggering of an endocytic process initiated by binding of the bacteria to specific receptors on the target tissue (3, 10). In this way, bacterial adhesins determine the host tissue distribution and the nature of the infective process. In addition, it has been well documented that pathogens often coordinately regulate the expression of virulence factors, often via environmental signals presented in the host (11, 12, 30, 31).

N. gonorrhoeae is an obligate human pathogen. The lack of an animal model in which to study pathogenesis has slowed progress in the elucidation of both GC and host cell factors that are pertinent to virulence. Thus, pending development of a suitable animal model for GC infections, the alternatives of organ and cell line culture systems are used to determine the roles of various GC components involved in host cell interactions. A tissue culture invasion system for *N. gonorrhoeae*, using the human endometrial cell line Hec1B, was reported in 1988 (43). This system demonstrated the invasive capacity of GC and their ability to survive intracellularly. Piliated GC bound the target cells at two to three times the level of non-piliated GC binding; however, once bound to the target, both GC types invaded at the same rate. The most notable finding was the long time course necessary to achieve GC invasion. Most invasive bacteria enter their targets quickly following adherence, usually within 2 h. However, GC that were centrifuged onto target cells still required 6 to 8 h to achieve internalization, as measured by attainment of gentamicin resistance and confirmed by electron microscopy.

Previously we reported that GC converted to an invasive phenotype (Inv⁺ GC) during this time lag between contact and invasion (6). Preincubation of GC with glutaraldehyde-fixed Hec1B cells resulted in Inv⁺ GC that invaded Hec1B targets faster and at a 10-fold-higher level than GC preincubated in tissue culture medium alone (Inv⁻ GC). However, no increased adherence could be detected in piliated Inv⁺ GC. This enhanced Inv⁺ phenotype was not a means of selection for hyperinvasive variants; continued contact of GC with Hec1B was required for maintenance of the Inv⁺ phenotype. Conversion could be blocked by the addition of chloramphenicol (1 μ g ml⁻¹) during the induction incubation, implying the need for GC protein synthesis. GC invasion was also blocked by the addition of cytochalasin D, an inhibitor of actin polymerization. Thus, the actin cytoskeletal system is part of the host cell uptake mechanism.

Bacterial adherence to host tissues is a prerequisite for invasion. In the case of GC, adherence is also necessary to

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provide the induction signal, delivered by the host cell, that leads to the development of the enhanced invasive phenotype. The remarkable degree of host tissue specificity observed in preliminary studies led us to narrow our search for the Inv^+ induction signal to factors relatively specific for human reproductive tissues. Three lines of evidence led us to narrow our focus to the lutropin receptor (LHR). The first was the relatively high specificity of the LHR for human reproductive tissues and its ability to undergo receptor-mediated endocytosis following stimulation with its cognate hormones, human chorionic gonadotropin (hCG) and luteinizing hormone (LH). The others were GC invasion studies in a fallopian tube organ culture (FTOC) model of salpingitis and epidemiological studies.

A number of studies have used FTOC as a qualitative in vitro system to study the interaction of GC with the mixture of cell types found there. Initial studies showed that GC lipooligosaccharide (LOS) is toxic to the ciliated cells while the non-ciliated cells are the target for GC invasion (28). Invasion studies showed that continuous addition of hCG to FTOC decreased GC invasion while a brief 24-h pretreatment with hCG, followed by a 2-day invasion assay, led to a doubling of invasion compared to a control not subjected to hormone treatment. It was hypothesized that invasion is mediated by an hCG-like molecule produced by GC; thus, invasion was blocked by hCG in the continuously treated sample while the enhanced invasion in the 24-h-pretreated sample was due to rebound up-regulation of the LHR (14).

Epidemiological studies have shown that the incidence of GC PID is not random but rather is clustered in the first week of the menstrual cycle (9, 33, 45), suggesting that fluctuations in levels of the hormones that regulate the menstrual cycle might influence the susceptibility of these tissues to GC invasion. It has recently been discovered that cells of the human fallopian tube express the LHR. The LHR is internalized following ligand binding and is known to undergo down-regulation in response to hCG or LH stimulation (20, 39). The expression of this receptor in the fallopian tube varies within the menstrual cycle from a nominal level in the proliferative phase, which drops following the mid-cycle LH spike and then rises through the secretory phase (21, 41). Thus, the increased expression of the LHR immediately precedes the temporal increase observed with GC PID. We hypothesize that this coincident pattern is due to variations in GC virulence associated with hormone-induced changes in LHR numbers.

This paper presents data from our current studies on the contact-inducible enhanced invasion phenotype of GC, with emphasis on the necessary host cell factors and their function in GC invasion of Hec1B cells. We have shown that of the nine cell lines initially tested, only human cells from the reproductive tract could convert GC to the invasive phenotype, though they did so to different degrees. We present evidence for the existence of two GC adhesins that interact with human host cell LHR; one is constitutively expressed, while the other is the unique contact-induced adhesin. We also show that invasion of Hec1B by Inv^+ GC is dependent on access to the LHR. We conclude with a proposed model of GC invasion of Hec1B cells in which the LHR plays a dual role as both the specific host cell surface feature that induces GC to convert to the Inv^+ phenotype and the specific uptake mechanism utilized by Inv^+ GC. Thus, the contact-inducible Inv^+ phenotype represents a set of adaptations which would target the upper reproductive tracts of women for complicated GC disease.

MATERIALS AND METHODS

Tissue culture cells. All cells were purchased from the American Type Culture Collection (Rockville, Md.) and maintained in RPMI 1640 medium plus 2 mM glutamine (Life Technologies, Grand Island, N.Y.) supplemented with 5 to 10% fetal bovine serum (FBS) (HyClone Laboratories Inc., Logan, Utah, or Atlanta Biologicals, Atlanta, Ga.) and 1 mM sodium pyruvate (Sigma Chemical Company, St. Louis, Mo.), at 37°C in 5% CO_2 unless otherwise stated. Cell lines were passaged every 5 days and discarded following approximately 20 passages. The cell lines used included A431 (a human epidermoid carcinoma cell line), ME180 (a human metastatic cervical epidermoid carcinoma cell line), C-33A (a human cervical carcinoma cell line), AN3CA (a human metastatic endometrial adenocarcinoma cell line), Hec1B (a human endometrial adenocarcinoma cell line), HeLa (a human cervical epitheloid carcinoma cell line), HEP-2 (a human larynx epidermoid carcinoma cell line), Chang (a human conjunctiva cell line), CHO (a Chinese hamster ovary transformant cell line), and MLTC-1 (a murine Leydig tumor cell line).

Preparation of induction and target monolayers. Induction monolayers were prepared by growing the particular host cells to >85% confluency (>48 h of growth) prior to fixation in 2.5% glutaraldehyde (grade II; Sigma Chemical Company) for 30 min at room temperature. Fixed monolayers were washed five times in Dulbecco's phosphate-buffered saline with calcium and magnesium, pH 6.5 (D-PBS+Ca/Mg), prior to use as induction monolayers. Target cells for adherence studies were plated into 48-well tissue culture plates (Costar Corporation, Cambridge, Mass.) at 10^5 cells per well. Invasion target monolayers were seeded into 24-well tissue culture plates (Corning Inc., Corning, N.Y.) at 2×10^5 cells per well. Targets cells were allowed to grow for 36 to 48 h prior to use in adherence or invasion assays, yielding >85% confluence.

Bacterial strains. *N. gonorrhoeae* F62 was used exclusively. Morphologically transparent, nonpilated variants were selected on Swanson's medium (44), and frozen stock cultures of these transparent type 4 colonies were maintained and used for all adherence and invasion assays unless otherwise noted. The opacity protein-negative (Opa^-) phenotype was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis of outer membrane preparations, and no differences in the patterns of outer membrane proteins of input and intracellular GC were observed (data not shown). GC were grown in GC broth or solid GC medium base (Difco Laboratories, Detroit, Mich.) with 1% Kellogg's supplement (19) at 37°C in a 5% CO_2 incubator.

Induction pretreatment. GC were pretreated by incubation at a multiplicity of infection (MOI) of approximately 50 on washed, fixed, confluent induction monolayers in RPMI 1640 plus 2 mM glutamine, 1 mM pyruvate, and 5 to 10% FBS supplemented with 0.02 mM FeCl_3 (complete medium) or in complete medium alone. The requirement of iron supplementation for GC invasion of Hec1B cells was previously established (6, 43). Contact between GC and fixed induction monolayers was achieved either by centrifuging the GC onto induction monolayers at $800 \times g$ for 10 min prior to incubation or by gently rocking the infected induction monolayers (4 oscillations min^{-1}) during the incubation at 37°C in a 5% CO_2 incubator. Following the desired induction period, the infected monolayers were washed three times in D-PBS+Ca/Mg, scraped into complete medium without FeCl_3 , vortexed vigorously for 1 min, and spun briefly to pellet the induction monolayer, and then the GC-containing supernatant was collected. This suspension was standardized to a concentration of approximately 8×10^6 GC ml^{-1} in complete medium with FeCl_3 (0.02 mM) and chloramphenicol ($1 \mu\text{g ml}^{-1}$) unless otherwise noted. GC pretreated in complete medium alone were simply vortexed vigorously and appropriately diluted to the standard infecting inoculum. These are defined as Inv^- GC. These pretreated GC inocula were added at either 0.25 ml per well of a 48-well plate in adherence assays or at 0.5 ml per well of a 24-well plate in invasion assays. GC concentrations were determined for each suspension prepared (input CFU).

Adherence assays. Adherence to target Hec1B cells was determined by metabolically labeling GC during the induction phase, using methionine-deficient RPMI 1640 (Life Technologies) with $15 \mu\text{C}$ of $\text{Expres}^{35}\text{S} \text{ ml}^{-1}$, a mixture of [^{35}S]methionine and [^{35}S]cysteine (New England Nuclear, Boston, Mass.). Labeled GC were prepared from induction monolayers as detailed above. Inv^- GC were washed three times in D-PBS+Ca/Mg prior to dilution to a standard concentration of infecting inoculum. The 48-well plates with infected cells were incubated with rocking at 37°C in a 5% CO_2 incubator. At the desired time points, targets cells were washed three times in D-PBS+Ca/Mg and lysed in 1% SDS, and then adherent GC were determined by scintillation counting.

Competitive adherence assays. Hec1B-induced GC (Inv^+ GC) or Inv^- GC were labeled with [^{35}S]methionine for a 4-h induction period. In addition, large numbers of unlabeled Inv^- GC were prepared. Fixed amounts of [^{35}S]methionine-labeled Inv^+ or Inv^- GC were mixed with increasing amounts of unlabeled Inv^- GC, the latter being a competitor for common binding sites on the Hec1B targets. These GC mixtures were used in a standard adherence assay. After 45 min of incubation, the samples were washed and adherent GC were determined by scintillation counting. For the dual competition assay, 100 IU of hCG (USP; 5,000 IU ampule $^{-1}$; ICN Pharmaceuticals, Inc., Costa Mesa, Calif.) was added to each well immediately prior to the addition of GC.

Invasion assays. GC invasion was determined by standard gentamicin resistance assays. Pretreated GC were incubated on Hec1B targets cells (approximate MOI, 10) for the desired period of time at 37°C in a 5% CO_2 incubator and

FIG. 1. Effect of host cell type on induction of Inv^+ phenotype. Confluent monolayers of various eucaryotic cell lines were fixed in 2.5% glutaraldehyde, washed, and incubated with GC for 4 h. Adherent GC were washed and used to invade Hec1B targets for 8 h. Target cells were treated with gentamicin to kill extracellular GC, removed from the wells, vortexed vigorously to release the intracellular GC, and plated to determine the number of gentamicin-resistant GC. The results from triplicate wells were averaged and are presented as the ratios of gentamicin-resistant CFU (O) to input CFU (I) + the standard errors of the means.

washed three times in D-PBS+Ca/Mg, and complete medium with gentamicin ($20 \mu\text{g ml}^{-1}$) was added for 90 min to kill extracellular GC. Target monolayers were washed two times in D-PBS+Ca/Mg, resuspended in 0.5 ml of 0.5 mM EDTA in D-PBS (pH 6.5), vortexed vigorously to release intracellular GC, and plated to determine the number of gentamicin-resistant GC (output CFU). For the competitive invasion assays, the denoted amount of hCG ($5,000 \text{ IU ampule}^{-1}$; ICN Pharmaceuticals, Inc.) was added immediately prior to the addition of GC.

LHR down-regulation studies. To achieve LHR down-regulation in Hec1B cells, hCG ($5,000 \text{ IU ampule}^{-1}$; ICN Pharmaceuticals, Inc.) was added to established (>24-h-old) Hec1B cultures at various concentrations overnight. These Hec1B monolayers were washed three times with D-PBS+Ca/Mg to remove any remaining hCG prior to fixation for use as induction monolayers or targets. For down-regulation studies using MLTC-1 cells, highly purified hCG (NICHHD CR 127) or LH (lot AFP9395A), provided by the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases, was added to established monolayers at 50 ng ml^{-1} in complete medium containing 1% bovine serum albumin (Sigma Chemical Co.) in place of FBS. These monolayers were washed free of FBS, incubated 18 h in the presence of hormone, and washed three times in D-PBS+Ca/Mg prior to fixation with glutaraldehyde.

Presentation of data. Unless otherwise stated, data points represent the means of triplicate determinations. Each experiment was repeated at least twice, and representative results are presented in the figures. Comparisons of various test conditions were achieved by using the paired Student's *t* test.

RESULTS

Screening cell lines for the ability to yield Inv^+ GC. To identify host cell factors necessary to convert GC to the enhanced invasion phenotype, a variety of eucaryotic cell lines were used as induction monolayers. Following a 4-h induction, the resulting invasive capacities of the adherent GC from these various monolayers were determined in an 8-h invasion assay. We found that contact-mediated GC induction is host cell specific, as only (but not all) cells from the human reproductive system provided the necessary signal to generate Inv^+ GC, though to different degrees. Human cells from nonreproductive tissues did not convert GC to the Inv^+ phenotype; invasion ratios were similar to that observed with GC pretreated by incubation in tissue culture medium alone (Inv^- GC). Among the reproductive cell lines capable of inducing the invasive phenotype, there were two- to fourfold differences in the proportion of the GC population converted to the Inv^+ phenotype, although all yielded higher invasion ratios than those obtained with nonreproductive cell lines (Fig. 1).

FIG. 2. Comparison of Inv^+ and Inv^- GC adherence. In this adherence time course, a standardized inoculum of ^{35}S -labeled GC was added to target Hec1B cells. At specific time points, target cells were washed to remove non-adherent GC and lysed in 1% SDS, and numbers of adherent GC were determined by scintillation counting. The Inv^+ GC (black bars) adhere to Hec1B faster and to a higher level than Inv^- GC (grey bars). The results from triplicate wells were averaged and are presented as percent adherence (output CPM/input CPM) + the standard error of the mean.

Adherence of GC to Hec1B cells. We quantified the effects of hCG on both adherence and invasion by GC by using the Hec1B system. Previously this laboratory reported that Inv^+ GC did not exhibit enhanced adherence to Hec1B cells (6). However, by eliminating the contributions of pilin, a potent GC adherence factor, we observed a twofold increase in adherence of Inv^+ GC to Hec1B cells (Fig. 2). Addition of exogenous LOS significantly decreased the observed binding of both Inv^+ and Inv^- GC. However, its effect on noninduced GC was consistently greater, with nonpilated Inv^+ GC exhibiting 15 times the adherence of nonpilated Inv^- GC in the presence of exogenous LOS (data not shown). The effect of hCG itself on the adherence of GC to Hec1B target cells is presented in Fig. 3. Adherence of both Inv^+ and Inv^- GC was suppressed, in a dose-dependent manner, by hCG. This suppression, while different in magnitude for Inv^+ GC, demonstrated the same

FIG. 3. Effects of hCG on adherence. Various amounts of hCG were added to Hec1B cells simultaneously with ^{35}S -labeled Inv^+ GC (black bars) or Inv^- GC (grey bars). At 2 h, the numbers of adherent GC were determined as described in the text. While the magnitude of hCG inhibition of Inv^+ GC adherence was greater, binding by both GC phenotypes was inhibited to the same percent by hCG. These representative results are averages of data for triplicate wells and are presented as percent adherence (output CPM/input CPM) + the standard error of the mean.

FIG. 4. Inv^- GC competitive adherence assays. In these adherence assays, fixed amounts of ^{35}S -labeled (*) Inv^+ GC (diamonds) or Inv^- GC (circles) were mixed with increasing amounts of unlabeled Inv^- GC as a competitor for adhering to common binding sites on Hec1B cells. These GC mixtures were incubated with Hec1B cells for 45 min, washed three times, and lysed in 1% SDS, and then adherence was measured by scintillation counting. The results indicate that Inv^+ GC bind to Hec1B by a mechanism not used by Inv^- GC. These representative results are the averages of data for triplicate wells and are presented as percent adherence without competitor $\pm$ the standard error of the mean. The MOI ratio refers to number of competing unlabeled GC divided by the number of ^{35}S -labeled GC, relative to the number of target cells.

FIG. 5. Dual competitive adherence assays. In these adherence assays, hCG was added simultaneously with GC as an additional competitor. Adherence was measured as detailed in the text. These results demonstrate that the unique Inv^+ GC (closed diamonds) binding mechanism is completely eliminated by the addition of 100 IU of hCG/well (open diamonds) while the adherence of labeled (*) Inv^- GC is not significantly affected by addition of hCG (no hCG, closed circles; with hCG, open circles). These representative results are the averages of data from triplicate wells and are presented as percent adherence without competitor $\pm$ the standard error of the mean. The MOI ratio refers to the number of competing unlabeled GC divided by the number of ^{35}S -labeled GC, relative to the number of target cells.

percentage decrease in both induced and noninduced GC. This implies that hCG competes with a constitutive GC binding mechanism but leaves unresolved the issue of its effect on the inducible enhanced adherence process.

Competitive binding assays indicate that the contact-induced adhesin is unique to Inv^+ GC and interacts with the LHR. Time course studies showed that binding of Inv^+ GC to Hec1B target cells occurred more rapidly and reached a higher steady-state level of approximately double that observed with Inv^- GC (Fig. 2). This implies that a highly specific binding mechanism is present on Inv^+ GC. The conversion to an Inv^+ phenotype requires *de novo* protein synthesis (6); thus, the new adhesin could be a specifically induced adhesin protein, an enzymatically modified constitutive adhesin, or a constitutively expressed adhesin that is subjected to simple up-regulation. By using a competitive binding assay, we tested whether this enhanced adherence is due to new adhesin or to increased expression of a constitutive adhesin. GC that bind by the same surface features, with equivalent binding affinities, should equally compete with each other for target cell binding sites. In this experiment, a fixed amount of ^{35}S -labeled Inv^+ or Inv^- GC was mixed with increasing amounts of Inv^- GC. The binding of ^{35}S -labeled Inv^- GC was significantly suppressed by the addition of unlabeled Inv^- GC. In contrast, trace amounts of Inv^+ GC maintained a higher level of binding at a greater than 100-fold excess of Inv^- to Inv^+ GC (Fig. 4). This implies that the type of adhesin(s) and/or the binding affinity of the adhesin(s) present on the surface of Inv^+ GC is significantly different from that present on Inv^- GC. The addition of hCG (100 IU per well) to the competitive adherence assay as a second agent completely eliminated the enhanced binding of Inv^+ GC, with a nominal effect on Inv^- GC adherence (Fig. 5). These data strongly implicate LHR as being both the high-affinity binding site specifically used by Inv^+ GC and the binding site for a constitutively expressed GC adhesin.

LHR down-regulation affects both conversion to and uptake of Inv^+ GC. The events that culminate in GC invasion can be divided into induction of the Inv^+ phenotype, adherence to

host cell uptake components, and subsequent internalization by target cells. Incubation of GC on a fixed Hec1B monolayer induces the Inv^+ phenotype (6). Inv^+ GC can be recovered and used to inoculate fresh Hec1B target cells for invasion studies (6). This procedure allows the separation of induction of the Inv^+ phenotype from internalization of GC by target cells. By using hCG to stimulate down-regulation of the LHR, we studied the effects of receptor numbers on induction po-

FIG. 6. Effect of hCG pretreatment of Hec1B induction monolayers or Hec1B targets on GC invasion. Established Hec1B monolayers were either incubated overnight with 50 to 100 IU of hCG ml^{-1} (hCG pretreated) or not treated (no hCG) and used for induction monolayers as detailed in Methods and Materials. Adherent GC from these Hec1B monolayers as well as GC grown in complete RPMI, as a control for the environmental conditions found in the induction plates (RPMI control), were used in invasion assays on Hec1B targets (black bars). These results show that hCG pretreatment of Hec1B led to an approximate twofold decrease in the ability of these fixed cells to convert GC to the Inv^+ phenotype. Similarly, Hec1B cells plated for use as invasion targets were treated with hCG (75 IU ml^{-1}). Following overnight incubation, these Hec1B cells were washed in PBS and used as invasion targets for GC from the three induction conditions described previously. Invasion of hCG-pretreated targets by GC from induction monolayers decreased approximately twofold (grey bars) compared with their ability to invade untreated Hec1B targets (black bars). However, hCG pretreatment of target cells had no effect on invasion by RPMI control GC (Inv^- GC). The results are averages of data from triplicate wells for over six experiments. Invasion is expressed as a percentage of the value for no-treatment controls (treatment [percent gentamicin-resistant CFU]/no treatment [percent gentamicin-resistant CFU]).

tential and target cell internalization in this two-stage invasion protocol (Fig. 6). GC induced on hCG-treated Hec1B monolayers (prior to fixation) invaded target cells at only half the level of GC induced on untreated monolayers ($P < 0.001$). Treatment of target cells with hCG prior to inoculation led to a twofold drop in invasion by GC induced on an untreated monolayer ($P < 0.003$), with little effect on invasion by Inv^- GC ($P > 0.3$). These effects of hCG pretreatment on LHR numbers are independent, as when both induction monolayers and targets were treated, a fourfold drop in invasion by GC occurred. These results suggest that the LHR plays two distinct and critical roles in GC invasion: induction of the Inv^+ phenotype and internalization of Inv^+ GC by the target cell. Competitive invasion studies, in which hCG and GC were added simultaneously, showed a significant decrease in Inv^+ GC invasion ($P < 0.001$) and no effect on Inv^- GC invasion ($P < 0.2$, paired Student's t test) (data not shown). This substantiates the need for access to LHR in Inv^+ GC internalization by Hec1B target cells.

An additional study was performed to confirm the role of LHR as the induction signal presented by the host cell to convert GC to the Inv^+ phenotype. The LHR on Hec1B cells has never been characterized concerning its response to hCG stimulation, especially with regard to receptor down-regulation. Since LHR are highly conserved among species, we tested the ability of the LHR of a murine cell line, MLTC-1, to serve as the induction signal. The extracellular domains of human and murine LHR are 84% identical at the protein level, with the differences being scattered along the 366-amino-acid extracellular region (41). These Leydig tumor cells bear LHR on their surfaces and, in response to either LH or hCG stimulation, secrete progesterone. The hormone-responsive cyclic AMP (cAMP) system is intact in this tumor cell line, and LHR are known to undergo internalization, with virtually complete loss from the cell surface, when incubated in the presence of 1 to 2 nM concentrations of LH or hCG for greater than 12 h (23, 36, 37, 41). The untreated MLTC-1 monolayers were nearly as effective as Hec1B cells at converting GC to the Inv^+ phenotype, with the level of induction being statistically significantly different from that in complete medium alone ($P < 0.02$). Treatment of MLTC-1 cells with nanomolar concentrations of either LH or hCG nearly eliminated their induction potential, with invasion indices being indistinguishable from those of GC incubated in complete medium alone ($P > 0.2$) (Fig. 7). This confirms the results of previous LHR down-regulation studies in Hec1B cells and supports the theory that GC require access to LHR for conversion to the Inv^+ phenotype.

DISCUSSION

Current studies clearly demonstrate that GC invasion is a multifactorial process (6, 18, 32) involving pilin (17), Opa proteins (2, 15, 24, 50), and LOS (25, 40, 46). The number of reported mechanisms of GC invasion of nonphagocytic cells probably represents both the high degree of adaptation of GC to very specific sites on their human host and the need for different adaptations during different stages of infection. These multiple adaptations of GC are confounded even further by the variety of host cell lines used in invasion assays, each with its own set of surface features. The remarkable degree of cross talk between bacteria and their host cells necessitates the study of invasion determinants in terms of their matched host cell receptors. Recent studies have identified specific host cell features that interact with Opa proteins, including sulfated heparin proteoglycans (47) and CD66a (7, 48, 49). Both of these cell receptors are up-regulated in response to cytokines and

FIG. 7. Ability of MLTC-1 to induce the Inv^+ phenotype. Fixed monolayers of Hec1B, MLTC-1, and MLTC-1 cells pretreated with 50 ng of either hCG or LH were used as GC induction monolayers, as detailed in Methods and Materials. Adherent GC from these four monolayer types and GC grown in complete RPMI medium were used to invade Hec1B targets. Adherent GC from MLTC-1 cells were as invasive as adherent GC from Hec1B cells. Treatment of MLTC-1 with nanomolar concentrations of either hCG or LH, conditions known to severely down-regulate LHR expression in MLTC-1 cells, virtually eliminated the ability of MLTC-1 to convert GC to the Inv^+ phenotype. Each datum was expressed as an invasion index relative to the RPMI control invasion level (sample [percent invasion]/RPMI [percent invasion]).

may enhance adherence and invasion of GC at sites of inflammation. In addition, the asialoglycoprotein receptor can interact with nonsialylated GC LOS. However, as most GC are sialylated in the human host (26, 34), the role of this host-GC interaction in human disease is uncertain. All of these studies involved the relatively constitutively expressed GC surface structures, although LOS and Opa proteins undergo both phase and antigenic variation. In contrast, this study focused on the set of GC adaptations that arise following contact with human reproductive cells expressing LHR and their effects on the early invasion events of host cell adherence and entry.

By using competition adherence studies, we have established the existence of both constitutively expressed (Inv^- GC) and contact-induced (Inv^+ GC) GC adhesins that interact with LHR. The uniqueness of the Inv^+ GC LHR adhesin was demonstrated by the inability of Inv^- GC to competitively inhibit the binding of Inv^+ GC to Hec1B cells, while the specificity of LHR was shown by the ability of hCG to completely abolish this enhanced Inv^+ GC adherence. A role for this contact-induced adhesin in GC invasion was implied by the ability of hCG to specifically inhibit Inv^+ GC invasion with a minimal effect on Inv^- GC uptake. By receptor down-regulation studies, we have shown that both conversion to and uptake of Inv^+ GC are dependent on access to LHR.

Based on this body of evidence, we propose the following model for the invasion of Hec1B cells by GC (Fig. 8). Upon contact with Hec1B cells, GC adhere to the cell surface by multiple mechanisms, including a constitutively expressed adhesin that recognizes LHR. Here Inv^- GC weakly and transiently bind to the receptor, a process that is inhibited by exogenous hCG. This interaction with LHR induces the conversion of GC to the Inv^+ phenotype. These Inv^+ GC can now bind to the target cells via a highly specific interaction with the LHR at a site other than that used by Inv^- GC, as demonstrated by the competitive adherence assays. This specific binding of Inv^+ GC to LHR stimulates receptor-mediated endocytosis and internalization of the Inv^+ GC.

The proposed model suggests that this is an organism that

FIG. 8. Proposed model of neisserial invasion of Hec1B cells. I. The gonococci come into the proximity of the LHR and engage in low-affinity binding to this receptor. This association may be mediated by a variety of surface components, including Opa proteins and/or 3F11 LOS. The nature and the actual site of this association with the LHR are unknown. II. Contact with the LHR induces the expression of a new GC surface adhesin. III. The induced adhesin mediates high-affinity binding to the LHR. The nature and the actual location of this binding site on the receptor are unknown. IV. The association of the induced GC with the high-affinity binding site on the LHR triggers the internalization of the receptor and results in the specific uptake of the induced GC in membrane-bound vesicles.

has developed a highly specific adaptation to its obligate host. By linking expression of specific virulence factors to exposure to the host uptake machinery, *N. gonorrhoeae* has evolved an extremely efficient regulation scheme by which it produces the induced adhesin/invasin only when it can be utilized.

The human LHR is a G-protein-coupled receptor found in gonadal tissue, uterine endometrial and myometrial compartments, fallopian tubes, decidua, placenta, fetal membranes, and blood vessels of the uterus, umbilical cord, and brain (8, 13, 21, 22, 38, 41, 42). It is a single polypeptide of 699 amino acids (41), of which approximately half (363 amino acids) are extracellular. The transmembrane region (amino acids 364 to 627) crosses the membrane seven times. It is believed that the intracellular tail (amino acids 628 to 699) and loops are responsible for interactions with G_s units, which act on adenyl cyclase to increase cAMP levels following hormone stimulation (41).

Regulation of the LHR is not well characterized. The gonadal LHR is known to undergo desensitization and down-regulation following hormone binding and G_s activation. These mechanisms include receptor-ligand internalization and degradation along with degradation of receptor-specific mRNA (16, 20, 35, 39). The level of LHR expression in the uterus and fallopian tubes is known to vary with the menstrual cycle, reaching peak levels at the beginning of menses (21, 41).

The gonadal LHR plays a major role in steroidogenesis. In addition, the LHR has been found in the blood vessel endothelium; studies of this tissue have discerned a transporter function for LHR for the delivery of the relatively large glycoprotein hormone from the bloodstream to its target tissues (13). This study found that both labeled hCG and LHR antibodies were taken up in coated pits and transcytosed to the abluminal surface of the rat testicular microvasculature endothelium. The role for the LHR in the endometrial lining has not been directly addressed. The endometrial epithelium does not have a role in steroid production in the female. Its most likely role is that of a transporter to facilitate the uptake of fetally derived hCG for interaction with the mother's endocrine system to ensure the maintenance of the uterine lining. The level of uptake of hCG by the mother is high, as a woman's serum level of hCG can reach 250 IU ml^{-1} in the first trimester (5). This strongly

implies the existence of an efficient transport system that utilizes a nondegradative pathway to preserve the bioactivity of the hormone. GC capable of using such a transport system would be highly selected for, as bacteria utilizing this uptake mechanism would be protected from degradative intracellular pathways in their transit through the mucosal barrier.

The utilization of the LHR could also account for some of the difficulty in developing an animal model for GC PID. While most mammals use LH for steroid regulation, only primates and equines use fetally derived CG to maintain the uterine tissues during pregnancy. Since other mammals do not use CGs, they do not have this receptor in their lower reproductive tracts.

This proposed mechanism of GC uptake and transcytosis could also explain the pattern observed in FTOC invasion studies. These studies revealed that GC attached to the microvilli and, within 24 h, were found in membrane-bound vacuoles inside the nonciliated cells. These vacuoles, with many GC inside, then appeared to migrate to the basolateral surface, where the GC were exocytosed into the submucosal layer (29).

The most intriguing aspect of this model is its correlation with clinical observations. The higher incidence of invasive disease in women than in men could be due to the lack of LHR in the lower reproductive tracts of men. The incidence of both disseminated GC infection and GC PID is closely associated with the onset of menses. If this correlation was due to either enhanced access to the upper reproductive tract or increased susceptibility of these tissues to infection at menses, then all causes of bacterial PID would show the same type of incidence clustering. This is not the case (9, 33, 45). The relatively recent identification of LHR in fallopian and uterine tissues and the determination of the role of this receptor at these sites provide evidence of an uptake mechanism specific for female reproductive tissues. The increase of LHR numbers through the secretory phase explains the increasing probability of GC contact and conversion to the invasive phenotype occurring late in the menstrual cycle. The time lag between peak LHR numbers and onset of clinical disease allows for phenotypic conversion of the GC and development of discernable disease.

Our current focus is on cloning the contact-inducible LHR adhesin for mutation studies to fully establish its role in GC invasion. In addition, we are examining the differential effects of Inv^- and Inv^+ GC on Hec1B cells. We are especially interested in determining whether Inv^+ GC exhibit any hCG-like effects on these cells. Since hCG stimulation initiates a cAMP signaling cascade in these cells, we are interested in studying the effects of these downstream events on GC invasion.

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