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Characterization of the urethra -associated mucosal immune responses to Chlamydia trachomatis in the male host.

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CHARACTERIZATION OF THE URETHRA-ASSOCIATED MUCOSAL IMMUNE RESPONSES TO *CHLAMYDIA TRACHOMATIS* IN THE MALE HOST

by

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A DISSERTATION

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ABSTRACT OF DISSERTATION
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Title Characterization of the Urethra-Associated Mucosal Immune Responses to *Chlamydia trachomatis* in the Male Host

Very little information exists about the immune responses to *Chlamydia trachomatis* (Ct) in males. This study was performed to characterize immune responses in the male urethra to the sexually transmitted disease (STD) agent Ct. Another aim of the study was to determine whether the origin of the more prominent components of the identified immune responses was epithelial cells by conducting *in vitro* studies. Previously published studies of Ct-infected murine animal models found increased levels of the Th1-associated cytokine, interferon-gamma (IFN-γ). In the present study performed in humans, we found levels of IFN-γ in urethral swab samples from Ct-infected males that were appreciably lower than expected when compared with the animal model reports. The primary components of the general and specific immune responses were increased levels of the proinflammatory cytokine IL-8 and total immunoglobulins, increased concentrations of chlamydia-specific IgA and IgG, and increased numbers of neutrophils.

To determine whether a source of the IL-8 was urethral epithelium, an *in vitro* primary urethral epithelial cell culture system was established. Primary epithelial cells were grown from readily available male urethral swab specimens and were characterized by transmission electron microscopy, morphological analysis, immunohistochemical stain-

ing for cytokeratins, and Western blots for cytokeratin 19. The primary cell culture characteristics were compared with those of a carcinoma-derived female urethral epithelial cell line, Hs769.T, that had not been previously described. Both the primary cells and the cell line could be infected with Ct. Infectious Ct elementary bodies from previously infected primary cells or from the cell line were capable of infecting other cell cultures of similar and dissimilar cell types. When compared with a currently used mouse fibroblast cell culture system, the human urethral epithelial cell line culture system showed marginally increased numbers of inclusion-forming units in primary, patient-associated Ct infections. These results established that these primary cells and the commercially available cell line were appropriate for passing Ct and that the cell line could be used as a possible replacement for animal cells currently used in most Chlamydia cell-culture laboratories. These newly evaluated cells (primary and cell line) provided an *in vitro* system for Ct infection studies.

Results of the *in vitro* Ct-infected cell culture experiments proved that the male stratified squamous urethral epithelium was not a major contributor to the increased levels of IL-8 in the locally sampled areas of the penile urethra. Examination of a Ct-infected prostate cell line (PC-3) and an embryonal kidney cell line (293) revealed no increased levels of IL-8.

DEDICATION

This work is dedicated to my wife, Phoebe, and our daughter, Emily. They may have suffered from time to time due to my absence during this educational pursuit; yet, it is I who would have suffered without their presence, their love, and their continued support in my life.

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URETHRAL CYTOKINE AND IMMUNE RESPONSES IN *CHLAMYDIA TRACHOMATIS*-INFECTED MALES

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PRIMARY URETHRAL EPITHELIAL CELLS DO NOT PRODUCE CYTOKINES AFTER EXPOSURE TO *C. TRACHOMATIS*

LIST OF ABBREVIATIONS

ADCC	antibody dependent cell-mediated cytotoxicity
APC	antigen-presenting cell
BALT	bronchus-associated lymphoid tissue
BCG	Bacille Calmette-Guerin
CMI	cell-mediated immunity
CMIS	common mucosal immune system
CTB	cholera toxin B
CTL	cytotoxic T lymphocyte
DEAE	diethylaminoethyl
EB	elementary body
ECL	enhanced chemiluminescence
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GALT	gut-associated lymphoid tissue
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
HBSS	Hank's balanced salt solution
HEV	high endothelial venule
HSP	heat-shock protein

ICAM-1	intercellular adhesion molecule-1
IEL	intraepithelial lymphocyte
IFN	interferon
ICE	interleukin-1β converting enzyme
IEL	intraepithelial lymphocyte
IgA	immunoglobulin A
IgG	immunoglobulin G
IL	interleukin
J-chain	joining chain
LFA	lymphocyte function-associated antigen
LGV	lymphogranuloma venereum
LPS	lipopolysaccharide
m	monomeric
M cell	microfold cell
MAC	membrane attack complex
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MALT	mucosal associated lymphoid tissue
MGV	mycostatin gentamicin vancomycin
MHC	major histocompatibility
MLN	mesenteric lymph node
MOI	multiplicity of infection
MOMP	major outer membrane protein

LIST OF ABBREVIATIONS (Continued)

MoPn	mouse pneumonitis
NALT	nasal-associated lymphoid tissue
NGU	non-gonococcal urethritis
NK	natural killer
OIF	oil immersion field
OPD	o-phenylenediamine
p	polymeric
PCR	polymerase chain reaction
PHUEC	primary human urethral epithelial cells
PID	pelvic inflammatory disease
PIgA	polymeric immunoglobulin A
PIgM	polymeric immunoglobulin M
pIgR	polymeric immunoglobulin receptor
PMN	polymorphonuclear
PP	Peyer's patches
RB	reticulate body
SARA	sexually acquired reactive arthritis
SC	secretory component
SIgA	secretory IgA
SLPI	secretory leukocyte protease inhibitor
SPG	sucrose phosphate glutamate
STD	sexually transmitted disease

TGF	transforming growth factor
VCAM	vascular cell adhesion molecule

INTRODUCTION

Descriptions of immune responses in humans have been conveyed best through the use of analogies and metaphors. A more often used metaphor presents the immune system as being in a continuous battle--a war. This war engages the army of the immune system against an ever-present enemy of potentially invasive pathogens that lie outside each human's intact borders. For the immune system to recognize these foreign invaders and effectively defend against them, the host has developed processes for identifying cells that are different from self cells and responses that counteract the efforts of the enemy. Countering the host's defensive measures, pathogenic microbes have established mechanisms and strategies that can subvert the immune response, or they can evade the immune system altogether, becoming stealth pathogens invisible to the host's defenses.

Aside from the immune system's offenses that are directed at pathogens, the body has also developed mechanisms that allow for the mutual existence of symbiotic and commensal organisms that yield benefits to both the commensals and the host. The discerning ability of the body to determine which organism is a commensal or a pathogen, that is, friend or foe, borders on the miraculous, yet lends itself to description and analysis. The field of immunology has evolved, and through its scientific advancements, it has helped to explain and describe the biological interplay involved between humans and their environment. Whether it is information that details the activities of the immune machinery that aids in warding off offensive maneuvers from pathogenic bacteria or information about mutually beneficial outcomes from the co-existence of commensals and

man, the biological discipline of immunology has broadened our understanding of the human immune system. Immunology has also revealed many facts about microbial pathogenesis, symbiotic relationships, inflammation, and immunological non-responsiveness, also called tolerance.

The human basic immune system has at its disposal an arsenal of weaponry that protects in both offensive and defensive manners. Offensively, it may act by coating non-pathogenic bacteria with antibodies that prevent bacterial adherence to the host and acts to impede their movement. Pathogenic invasive bacteria, are covered by specific antibodies that may also serve to identify the enemy as foreign, marked for destruction. Defensively, portions of the immune system may attack invaders. Macrophages devour and eliminate pathogens. Natural killer (NK) cells obliterate the offender through anti-body-dependent cell-mediated cytotoxicity (ADCC). Cytotoxic T lymphocytes (CTL) are summoned to destroy an intracellularly infected host cell and the pathogen contained within. Other immunoregulatory components may be invoked by the mere presence of the enemy's outer coat, such as lipopolysaccharide (LPS) that can non-specifically activate innate immune mediators. Complement can activate a cascade of components that include C5b, C6, C7, C8, and C9 to form the membrane attack complex (MAC) that punches holes in bacteria, resulting in their demise. All of these components are a part of the immune system's viable armamentarium that may be utilized to promote the welfare of an unsuspecting host.

The mucosal immune system and its relation to the male genital tract

The immune system has more components and related actions than the few that have been mentioned. Generally the immune system can be described as consisting of two

distinct compartments: the systemic and the mucosal compartments. The systemic compartment is identified with the immunocompetent cells from the lymph nodes, spleen, and bone marrow. The mucosa-associated compartment is identified with comparable immune system cells in addition to immunocompetent cells related with the external secretory glands.

The major component of the human immune system is the mucosal immune system. This system protects large surface areas (~400 m^2) through active surveillance by immune system cells. This area is much larger than the body's external surface area (~1.8 m^2) and has more lymphoid cells associated with it than with any other tissues in the body (56). Not only does the scope and size of the identified mucosal tissues and their closely related immunoregulatory cellular components make this the major part of the immune system, but the fact that we would not exist without the mucosal immune system makes its presence essential for life and its understanding of foremost importance.

The mucosal immune system is composed of integrated regions of mucosa-associated lymphoid tissues (MALT) that are defined by anatomical locations (9). These separate, anatomically-defined lymphoid tissues can be classified at a functional level as either inductive or effector sites. Inductive sites are capable of inducing immunity to any encountered foreign antigen in contrast to the effector sites where the production of secretory IgA (S-IgA) is present in response to antigens. Neither inductive nor effector sites of MALT have activities limited only to their respective regions.

The MALT sites are identified by a common feature: follicle-associated epithelium (12) also termed microfold (M) cells (65). These cells are found in the respiratory tract and are identified with the bronchus-associated lymphoid tissue (BALT) (7, 8). Gut-

associated lymphoid tissue (GALT) includes Peyer's patches (PP), the appendix, mesenteric and solitary lymph nodes, intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (41, 65, 66). The nasal-associated lymphoid tissues (NALT) contain the adenoids, palatine, oropharyngeal, and lingual tonsils that make up the Waldeyer's ring (53). Additional MALT includes the genitourinary tract, inner ear, and the salivary, lacrimal, and mammary glands.

Although the genitourinary tract is considered to be a part of the mucosal immune system, the associated genitourinary immune system exhibits characteristics distinctly different from those of the typical mucosal or systemic sites. An example is a lack of lymphoepithelial follicles in the genital tract. Another difference exists between the content and isotype of antibody in secretions of the genitourinary tract and other external secretions. In the female genitourinary tract, total levels of IgA and IgG antibodies are derived probably equally: from local production and plasma. In other secretions such as milk, tears, and saliva, higher levels of IgA than IgG are usually evident. Hormone levels in the female genital tract affect the distribution of immunocompetent cells, immunoglobulin (Ig) isotypes, and molecular forms (93). However, there is little information available that relates hormonal influences to associated immune mechanisms of the male genital tract.

Mucosal tissues are covered by epithelia that act primarily as a physical barrier. Nonetheless, these epithelia do more than just obstruct pathogens from gaining entry into the host. Epithelia cells actively secrete innate immune factors, such as, antimicrobial defensin peptides, lactoferrin, lactoperoxidase, mucins, lysozyme, complement components, and cytokines (20). These soluble factors display antimicrobial properties, and

mucin may help prevent movement past the epithelium. The mucosa-associated epithelia are also active in the transcytosis of Ig and may also be involved in antigen processing and presentation (20).

Under the environmentally exposed epithelium is the lamina propria with its cellular components that include B-lymphocytes, T-lymphocytes, CTLs, macrophages, NK cells, eosinophils, and mast cells. These epithelial covered, distinct mucosal tissues are interconnected with all other mucosal-associated components through the common mucosal immune system (CMIS) (52, 55, 70, 80). The CMIS allows for the induction of immune responses at one (inductive) site and for the effects of an antigen-induced immune response to be made manifest at distant and anatomically unrelated effector-associated mucosal sites. In women immunized with a nasal vaccine, elevated levels of vaccine-specific antibodies have been found in anatomically removed sites such as the vagina (75).

Even though mucosa-associated responses can be achieved in the genital tract of females, as demonstrated by intranasally administered vaccines, neither the male nor the female genital tract have organized lymphoid follicles equivalent to those found in the Peyer's patches of the gut. This means that the genital tract in humans does not have identified inductive sites and lacks the ability to induce immune responses that can distribute antigen-sensitized effector cells such as B and T lymphocytes to distant and anatomically unrelated sites of the mucosal immune system. Lymphoid aggregates have been well described in the female uterine stratum basalis (95); nonetheless, these structures appear to be of a different organization than what is usually encountered in other mucosa-associated tissues. CMIS effector-like sites are located in the genital tracts of both sexes because of the presence of IgA plasma cells beneath secretory component (SC)-expressing

epithelial cells and S-IgA that is found through sampling of the lumen at respective sites. Because of this, the female genitourinary tract, and most likely the male tract too, are capable of mounting a mucosa-associated immune response to locally expressed antigens.

The mechanisms involved in the induction of immune responses in the GALT have served as a reference to aid in explaining the molecular and cellular interactions in other MALT compartments (41, 54). At mucosal surfaces environmental antigens are abundant. Approximately 10^{14} bacteria are located within the gut lumen of the large intestine (79). These bacteria and environmental antigens are subject to immune surveillance that involves a balance of microbial detection so that pathogenic antigens are recognized and immune responses generated against them, while other antigens, particularly of food origin are tolerated and under normal conditions do not induce an inflammatory response. Active uptake of these antigens for sampling is carried out through specialized lymphoepithelial structures in the gut tissue. These specialized structures include M or follicle-associated epithelia (FAE) cells that are located in the dome of the PP (11, 12, 65, 66, 82) and columnar epithelium (40). Once antigen has traversed the M cell, antigen processing and presenting cells, such as follicular dendritic cells, macrophages, or B cells, are recruited for action. These cells will process and present antigen peptides to the T cells located in specialized zones underlying the M cell (51). These responsive and engaged T-helper cells will next activate naive B-lymphocytes (located in B-cell zone germinal centers under the PP dome) to initiate switching of surface (s) IgM+ cells to sIgA+ cells (18, 47, 94). The IgA-committed B cells migrate from the GALT via lymphatics to the mesenteric lymph nodes (MLN). Next, the B cells continue to move through the thoracic duct (29, 31-33, 54, 71), into the systemic circulation, and ultimately home to mucosal effector

sites. Then B cells undergo terminal differentiation through the influence of local cytokines such as IL-5, IL-6, IL-10, and TGF-β (52, 54, 81). The destinations for these effector B cells may be the lamina propria of other mucosal sites.

Most human mucosal secretions contain IgA antibodies that predominate all other isotypes (IgE, IgG, and IgM). However, an exception exists in the male and female genital tract. Here, the IgG isotype levels are slightly higher than those of IgA. Structural differences in the IgA exist in the two compartments: plasma IgA exists primarily as a monomeric (m) IgA, while the IgA associated with secretions is most frequently polymeric (p) IgA.

In humans, IgA consists of two subclasses, IgA1 and IgA2. These subclasses are present in all mucosal secretions and can be found in the plasma. The levels of IgA1 subclass in plasma are higher than IgA2 (23). Structurally, an additional 13-amino acid segment exists in the hinge region of the IgA1, but not the IgA2 molecule (58). This extended hinge region also makes the IgA1 susceptible to IgA proteases secreted by bacteria such as *Neisseria gonorrhoeae*, *N. meningitidis*, and others (72). IgA1 has 3 to 5 *O*-linked glycans (containing *N*-acetylgalactosamine) that are not found in the IgA2 subclass (87). IgA1 has specificity for protein antigens, while the IgA2 appears to have a higher propensity to target LPS and lipotechoic acid and polysaccharide antigens (15, 46). In the human large intestine and the female genital tract, the numbers of IgA2-producing cells are slightly higher than or equal to the numbers of IgA1-producing cells (58). A possible explanation for the IgA2-producing cell increase over the IgA1-pro-ducing cells could be type of environmental antigen exposure at the two sites (58).

The IgA2 subclass has two allotypes that have been identified, A2m(1) and A2m(2). No functional properties have been ascribed to these allotypes. These two allotypes are characteristically distributed among different ethnic and racial populations. Caucasians, as well as Eskimos, Australian Aborigines, and Native American Indians have the A2m(1) allotypes in contrast to the African and African American populations, which have the A2m(2) allotype (89, 92).

Unlike the IgG isotype, the IgA isotype has the capacity to be spatially, physically, and molecularly different through arrangements with additional molecules of IgA. IgA can exist in a monomeric form, as well as in polymeric units: dimer, trimer, and a tetramer form (30). Multiple units of IgA require a joining (J)–chain and also serves as a marker for pIgA synthesis (34, 60). The J-chain is a glycoprotein of approximately 15-16 kDa linked by disulfide bridges to the Fc region of IgM or IgA.

Ig presented in the male genital tract secretions include IgA, S-IgA, IgG, and IgM. These isotypes have been found in pre-ejaculate, as well as seminal plasma. Different studies have reported disparate levels of Ig, which may be due to differences in specimen collection, Ig measurement protocols and their standards, and presence of proteolytic enzymes in the specimens that degrade Ig such as IgM (86).

The reported levels of S-IgA and IgG in seminal fluid have differences associated with them. Some investigators report more IgG than IgA in seminal fluid, while other reports state the opposite (57). However, in pre-ejaculate, higher levels of IgA were measured when compared to levels of IgG (personal observation). Levels from parallel measurements of plasma-derived protein (e.g., albumin, lactoferrin) and split ejaculate

show that the IgG present in seminal fluid is most likely derived from the circulation due to transudation while S-IgA is produced locally.

In normal tissues of the epididymis, seminal vesicles, or prostate, the inability to find Ig and SC by histochemical means and the lack of SC mRNA by Northern blot was reported by Brandtzaeg et al. (14). Yet, Anderson and Pudney (4) reported results of positive SC staining cells in the inflamed genital tract of HIV-infected men, indicating the presence of SC in all of these tissues. From normal tissues of the male urethra, the epithelial cells associated with the glands of Littré have stained positively for the presence of SC (68, 73), J-chain, IgA, IgG, and IgM plasma cells located near the same area. The presence of all of these components suggests that S-IgA is a product of the penile urethra because it is not found in other tissues in the male genital tract. Expression of high levels of pIgR has been found in the glands of Littré; therefore, these glands are associated with the transport of S-IgA into the male urethra (77).

At the mucosal sites, most IgA is usually present in the form of S-IgA. S-IgA is composed of 2-4 monomers with J-chain and an SC. The SC is a cleaved portion of the polymeric immunoglobulin receptor (pIgR) that remains once the pIgA or pIgM is secreted from the epithelial cells. The J-chain on polymeric IgA or IgM is required for interaction with pIgR.

The pIgR is essential for the transport of S-IgA through the epithelial cells. The pIgR is present on basolateral surfaces of mucosal epithelium that actively binds polymeric IgA or IgM before being internalized by the epithelial cells (see Fig. 1). Once the pIg is attached to the receptor it enters the cell within an endosome and is transcytosed to the apical surface where it is released. The released S-IgA is composed of pIg connected

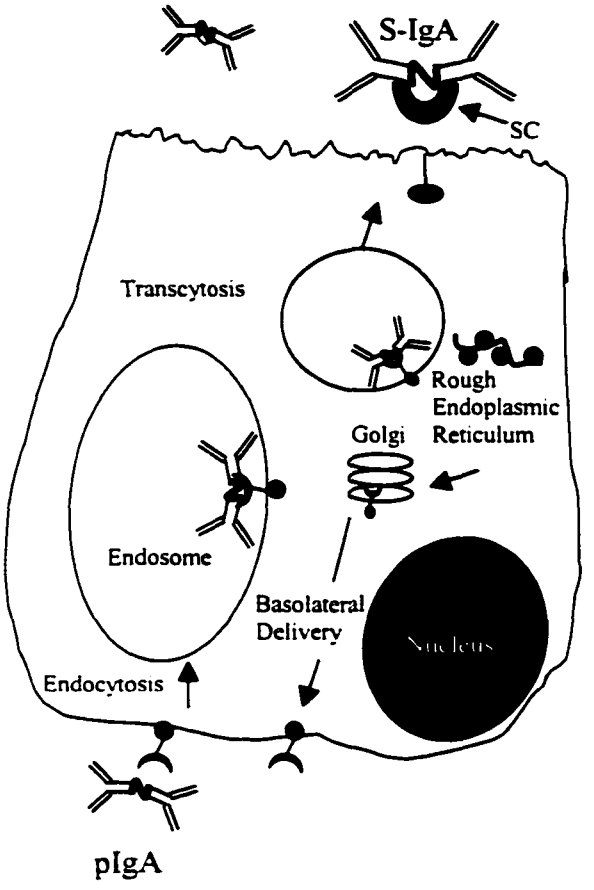

FIG. 1. Polymeric Ig receptor (pIgR) and transport of pIg. In the male urethra, pIgR is associated with the glands of Littré that aids in the secretion of S-IgA. This diagrammatic adaptation shows the process of pIgR generation and intracellular transport of pIg *via* the pIgR. Epithelial cells synthesize the pIgR within the rough endoplasmic reticulum. The pIgR then moves to the Golgi apparatus. At the trans-Golgi network, the pIgR is packaged into a vesicle that delivers it to the basolateral surface of the epithelial cell. Here pIgR is used to bind to the extracellular polymeric Ig. Next, the polymeric Ig produced by plasma cells adheres to the pIgR and is endocytosed into the cell and delivered to endosomes. The Ig is transcytosed by a series of vesicles in the cell from the basolateral side to the apical surface of the plasma membrane. At the surface, the pIg along with a portion of the pIgR is cleaved, and the resulting product is considered the S-Ig because of the secretory component attached to the polymeric Ig and J-chain.

with J-chain plus the truncated version of the pIgR, which is now SC, following cleavage of the pIgR-bound pIg.

Although IgA is present in any mucosal secretions, it does not function alone. A network that includes defense factors enhances the immune defense functions of IgA. Other locally produced substances are specialized in inter-cellular communications. One example of additional biological communications involves interaction with cytokines, chemokines, and their respective receptors. The chemical signals produced help to initiate, maintain, or terminate certain biological functions such as recruitment of effector T-lymphocytes or antigen presenting cells (APC). These three cell types appear to constitute >60% of the total MALT cellular components, but have additional support such as B cells.

Immunoglobulin A

The dominant Ig secreted into the external fluids is IgA. It predominates all other isotypes (IgE, IgG, and IgM) found in human mucosal secretions with the exception of isotypes in the male and female genital tract, where the IgG isotype is highest. IgA is found in both serum and in mucosal secretions. Even though they both exist within the body, they are most often considered products of mutually independent systems. Most of the IgA present in plasma is formed in the bone marrow (BM), but more IgA is produced in the GALT, which is the primary site of S-IgA synthesis. Here, approximately 40-60 mg/kg of IgA are generated per day, with the majority of this Ig found in external secretions (21, 59).

Mucosal Lymphocyte Homing

The most important mediators of specific immunity (see following section) are B and T lymphocytes. These cells have the capacity to move throughout the body using both the blood and lymph circulatory systems. Because the body's organs are distant from each other and must undergo constant surveillance, movement of lymphocytes is essential to maintain the integrity of the host and associated immune system. Lymphocytes recirculate through the blood and lymph to various lymphoid organs, such as the spleen, peripheral lymph nodes, and MALT. The lymphocytes are differentiated with preferential ability to migrate to specific areas, but following antigenic stimulation, they may have the capacity to be reprogrammed to home to tissue-specific areas (17).

Migration of the mucosal lymphocytes requires the involvement of mucosal addressins and cellular lymphocyte receptors. First, the mucosal-associated antigen-initiated B and T lymphocytes have to exit into the circulatory system and be further redistributed into the lymphoid organs or the area containing the inflammatory tissue. Mucosal lymphocytes, as well as neutrophils and macrophages, move in and out of circulation through venules by a process called extravasation. The vascular endothelial cells that line the walls of the blood vessels called high-endothelial venules (HEV) express mucosal addressin such as cell adhesion molecule-1 (MAdCAM-1) or glycosylation-de-pendent cell adhesion molecule-1 (GlyCAM-1) addressins on their cell surface. Some cell addressins are expressed constitutively on the cell surface, while others such as Mad-CAM-1 may be induced following trauma (36) or inflammation (2, 26). MAdCAM-1 is one of the ligands for the homing integrin $\alpha 4\beta 7$ that is expressed on B and T lymphocytes. Naive lymphocytes that express the peripheral lymph node homing receptor, L-selectin, have an affinity for bind-

ing the L-selectin homing receptor to vascular addressins such as GlyCAM-1 and CD34 on the HEV. Some evidence suggests that the integrins $\alpha 4\beta 1$ and $\alpha L\beta 2$, known as lymphocyte function-associated antigens-1 (LFA-1) and their respective vascular addressins, vascular cell adhesion molecule-1 (VCAM-1) and inter-cellular adhesion molecule-1 (ICAM-1) may play a part in homing of B and T lymphocytes to the genital tract (38). This is quite plausible due to the presence of both the ligand and the addressins in the genital tract (38).

Subset populations of lymphocytes express different homing receptors. This variation in receptors helps to add specificity to the immune response. The tissues also express specific addressins on the vasculature cell surface to recruit specific lymphocytes directed to antigen (23) at the effector site (48, 83). All of these activities are orchestrated and mediated through a series of secreted products such as cytokines, chemokines, plasma enzymes, and lipids.

Innate and acquired immunity

Two complementary units of the immune system act in different, but supportive ways that offer protection to the host. These two immune system units are referred to as innate and acquired immunity. Both are employed by the mucosal immune system as well as the general or systemic immune system.

At the first sign of danger, components of the innate immune system act on impulse in a non-specific manner (antigen independent) to defend against a broad spectrum of potentially harmful microorganisms that seek to breach the host's external barriers including skin and mucous membranes. These sentry components of the natural immune

system are always present and act early to defend the host. Components of the innate immune system include complement, phagocytic cells such as macrophages and neutrophils, natural killer cells, and macrophage-derived cytokines (tumor necrosis factor, α and β interferons) (1).

Components of the acquired immune system are engaged only after more information has been processed in order to respond in an appropriate manner. Information regarding the type of antigen that is present, how much antigen, and the antigen's primary mode of operation allow the acquired immune system to attack the offenders with more precision than the innate immune system. Another synonym for the acquired immune system is the specific immune system, which is composed of B lymhocytes, T lymphocytes, and lymphocyte-secreted products such as antibodies and cytokines (e.g., interferon-γ) (1).

Cytokines

Cytokines are soluble proteins that influence or modify several biological responses. They are secreted by various cells such as lymphocytes, monocytes, macrophages, epithelial cells, and NK cells, and can mediate and regulate the immunocompetent cells in the performance of specific immune-related functions. Cytokines can bind to specific receptors expressed on cell surfaces. These cytokine receptors are transmembrane proteins that mediate cell activation through tyrosine kinases, signal transducers, and activators of transcription (STAT) family proteins.

The following brief descriptions of eleven cytokines are included to allow comparisons and aid in explaining the relationship and roles that they play in immune responses to

Ct in the male urethra. These cytokines are pertinent for the studies of two of the three presented manuscripts.

IL-1

Precursors of the cytokine interleukin (IL)-1 exist as 31- to 33-kDa proteins. Through a series of enzymatic actions, the intracellular precursor form becomes the bio-active proform that exists as a 17-kDa protein in one of two distinctive forms, IL-1α or IL-1β (49, 85). This bioactive form is produced by many cell types, such as macrophages, monocytes, B cells, T cells, dendritic cells, and endothelial cells and acts as a mediator of acute inflammatory responses. Once IL-1 is induced and released to the affected area, inflammation results. Systematically, IL-1 acts to induce fever and can cause acute-phase protein production. IL-1 is required for the efficient production of IFN-γ.

IL-2

IL-2 is a 14- to 17-kDa protein that is primarily a product of T-helper lymphocytes (62). It has the capacity to act as an autocrine and paracrine growth factor. It is capable of promoting the T-lymphocytes from the G1 phase to the S phase of the cell cycle. IL-2 also acts as a growth factor for B cells (91) and is capable of inducing antibody synthesis from plasma cells. It acts in an autocrine manner to induce T cells to undergo clonal expansion as antigen-specific T cells. It also stimulates the growth of and cytolytic function of NK cells (37).

IL-4

The switching and differentiation of B cells to produce Ig can be initiated by IL-4 (27, 43, 74). This 20-kDa cytokine acts as a growth factor for mast cells, and its presence can increase the expression of VCAM-1 in activated vascular endothelium. In T cells, IL-4 has the capacity to act as a growth and differentiation factor. It is considered a Th2 type cytokine. IL-4 can also inhibit the activity of macrophages.

IL-6

IL-6 acts in an immuno-regulatory manner and as a proinflammatory protein. It is evident in many disease states. This approximate 26-kDa cytokine is produced by most nucleated human cells and activates major proteins of the acute phase responses *in vitro* and *in vivo*. IL-6 promotes terminal B cell differentiation and Ig production by plasma cells (42). IL-6 also regulates T-cell activation and increases, *in vitro*, IgA synthesis by Peyer's patch B cells. Fibrinogen production by hepatocytes is increased with higher levels of IL-6.

IL-8

The pro-inflammatory IL-8 is known as a chemoattractant cytokine (chemokine) and leukocyte activator that has a four-cysteine motif and is designated as a CXC chem.-okine. IL-8 is involved in recruitment of neutrophils to areas of inflammation (50) and infection. IL-8 is an 8-kDa cytokine that is secreted by activated monocytes, endothelial cells, and epithelial cells. The endothelial cells produce IL-8 as a means of recruiting neutrophils into the tissues where they are needed. By expressing IL-8 on the endothelial cell

surface, neutrophils attach to the endothelial cell surface and exstravasate from the vasculature into the tissues. *In vitro* studies show that many cell types stimulated with LPS, IL-1, or TNF-α will produce this cytokine.

IL-10

IL-10 is an 18-kDa glycoprotein that is produced by CD4+ T lymphocytes, activated B cells, and by some Th1 cells in humans. Cells with a Th2 profile most often produce IL-10 along with IL-4, IL-5, and IL-6. IL-10 is capable of inhibiting cytokine production by macrophages. It helps to down-regulate MHC class II expression and is efficient at inhibiting T-cell-mediated immune inflammation. IL-10 enhances the development of TGF-β-producing T cells in Peyer's patches because of its capacity to inhibit IL-12 production (81). The development of IgA-producing B cells is supported by IL-10.

IL-12

The heterodimer IL-12 is a 70-kDa cytokine that is composed of two subunits, p35 and p40. Both subunits are required for a functional IL-12 molecule. B cells, T cells, NK cells, and monocytes are capable of producing the p35 subunit, whereas activated monocytes and B cells make the p40 subunit. This cytokine is active in the effector phases of cell mediated immune (CMI) reactions. It can directly activate effector cells and can also regulate their development. IL-12 can stimulate NK-cells to produce IFN-γ and can help to differentiate the naive CD4+ T cells to a Th1 subset and the CD8+ T cells toward CTLs.

IL-18

IL-18 is a non-glycosylated peptide that is converted from an inactive pro-peptide form of approximately 24 kDa to an 18-kDa biologically active peptide form following proteolytic cleavage by the enzyme interleukin-1β converting enzyme (ICE). Macrophages, Kuppfer cells, keratinocytes, and osteoblasts express IL-18. This cytokine is important because it has a direct effect on the induction of IFN-γ, which is involved in immune defense. This is important due to the effects that IFN-γ has on the immune cells of the body.

INF-γ

The production of IFN-γ is basically the result of activated CD4+ Th1 cells, CD8+ CTL cells, NK cells, and monocytes/macrophages (10, 78). The production of this 34-kDa homodimeric glycoprotein is stringently controlled. Only the cytokines IL-2, IL-12, IL-18, and TNF-α can actively induce its synthesis. One of the more important activities of IFN-γ is the direct activation of macrophages for respiratory bursts (67) that result in anti-bacterial, anti-tumor, and anti-viral responses (10, 88).

Transforming Growth Factor-β

One of the more important cytokines that plays a major role in wound healing is the transforming growth factor (TGF)-β. This cytokine is responsible for stimulating cells to increase the production and deposition of extracellular matrix (ECM) in the healing tissues. It acts as a chemoattractant for monocytes, macrophages, and fibroblasts. Fibroblasts that are attracted to the area of inflammation or injury by TGF-β will help to pro-

duce the ECM proteins composed of collagens I, III, V, and fibronectin. In order to accelerate the healing process, this cytokine also acts to limit the degradation of the extracellular matrix by proteases. Strong evidence suggests that TGF-β induces IgA switch differentiation in B-cells. TGF-β is capable of inhibiting *in vitro* proliferation of many cell types such as T and B cells. Studies in rats have shown that with increased levels of TGF-β, over expression of fibrous tissue occurs (13) and fibrotic lesions in the liver and kidney may develop as a result.

Tumor Necrosis Factor-α

TNF-α, a 17-kDa protein found in serum, has the ability to cause necrosis of tumors *in vivo* and tumor cells *in vitro* (19) following bovine Calmette-Guerin (BCG) priming and endotoxin treatment of animals. Many cell types produce it, but most TNF-α comes from monocytes and macrophages. TNF-α is able to activate some acute-phase proteins, and it has multiple and differing effects on a variety of cells throughout the body.

The human male genital tract

If considered as a single continuous duct, the human male genital tract, including rete testis, epididymis, vas deferens, all associated ducts, and the penile urethra, would be approximately 13 m in length (3). Mucosal epithelium lines the whole duct, with variations along its length. These variations include different types of epithelium. At the distal end of the penis (includes the foreskin, meatus, fossa navicularis, and fossa terminalis), stratified squamous epithelia act primarily as a physical barrier that can be easily shed. Upon shedding, these squamous cells may take with them associated pathogens. At the

more proximal end of the duct, the epithelium includes columnar, cuboidal, and pseudo-stratified columnar epithelia (penile urethra, vas deferens, epididymis, rete teste, and prostate). Because of these extant differences in the types of epithelia present in the urethra, the immune system is likely to modify its responses due to the cellular structure alone (3). Both humoral and CMI responses are manifest when needed and are important for the defense against microbial invasion.

The simple epithelium has the capacity to develop glands that possibly secret mucin-rich products that may interfere with the attachment of pathogens. In the male urethra, mucin-producing periurethral glands are called the glands of Littré. Their secretions have been considered to be lubricant-like and also contain innate soluble antimicrobial substances that further prevent pathogen colonization of the male urethra.

From studies conducted for this dissertation, secretory leukocyte protease inhibitor (SLPI) has been identified for the first time as a product of the penile urethra. Along with other innate factors, SLPI is capable of deterring pathogen colonization.

CMI responses are important to the host defense of any mucosal area. The primary cellular responses in the penile urethra and associated genital tract tissue can be attributed to macrophages, T-lymphocytes (CD4+ and CD8+), and intraepithelial lymphocytes. Reports in some but not all studies state that the prostate contains both IgA- and IgM-positive plasma cells and that prostate tissue stains positive for the pIgR (3, 5, 24). In the present study, S-IgA was found at >1000 ng/ml in the transport media from urethral swabs in uninfected males.

The last study included in this dissertation compares a female urethral epithelial cell line to primary urethral epithelial cells obtained from the fossa navicularis in males.

Since direct comparisons were to be included in this dissertation, it was prudent to confirm that the two urethral epithelial cell types were of the same origin. If one relied on the teachings of the past, these two cell types could not be regarded as having the same ontogeny. Therefore, a more current overview of the male urethral ontogeny that is included in this thesis is used to validate the direct comparisons of the urethral cells that were taken from males and compared to a female urethral cell line.

For many years, authors of embryology textbooks have described that the ontogeny of the distal male urethra originated from a combination of endodermal and ectodermal cells. Recently, this dogma has been challenged by research findings indicating that the male urethra is strictly derived from endodermal origin extending from the urethral opening to the bladder (Fig. 2) (44). Since the origin of the female urethra had never been described in a similar manner, the idea that the male urethra had two distinct sources of origination would make it difficult to explain experimental results obtained with a female urethral cell line when those results were directly compared to experimental results obtained with primary male urethral epithelial cells. The results of experimental studies utilizing primary male urethral epithelial cells make direct comparison to the female urethral cell line relevant, plausible, and acceptable.

The basal surface of the epithelium has been identified as an area where S-IgA is generated; its presence in the urethra lumen helps to bind pathogens. Plasma cells are abundant in the male penile urethra tissue, somewhat analogous to the immunologically reactive female cervix. The male genitourinary tract is responsive to androgens and has defenses such as CD4+ cells, CD8+ cells, and Th1/Th2 cytokine production against path-

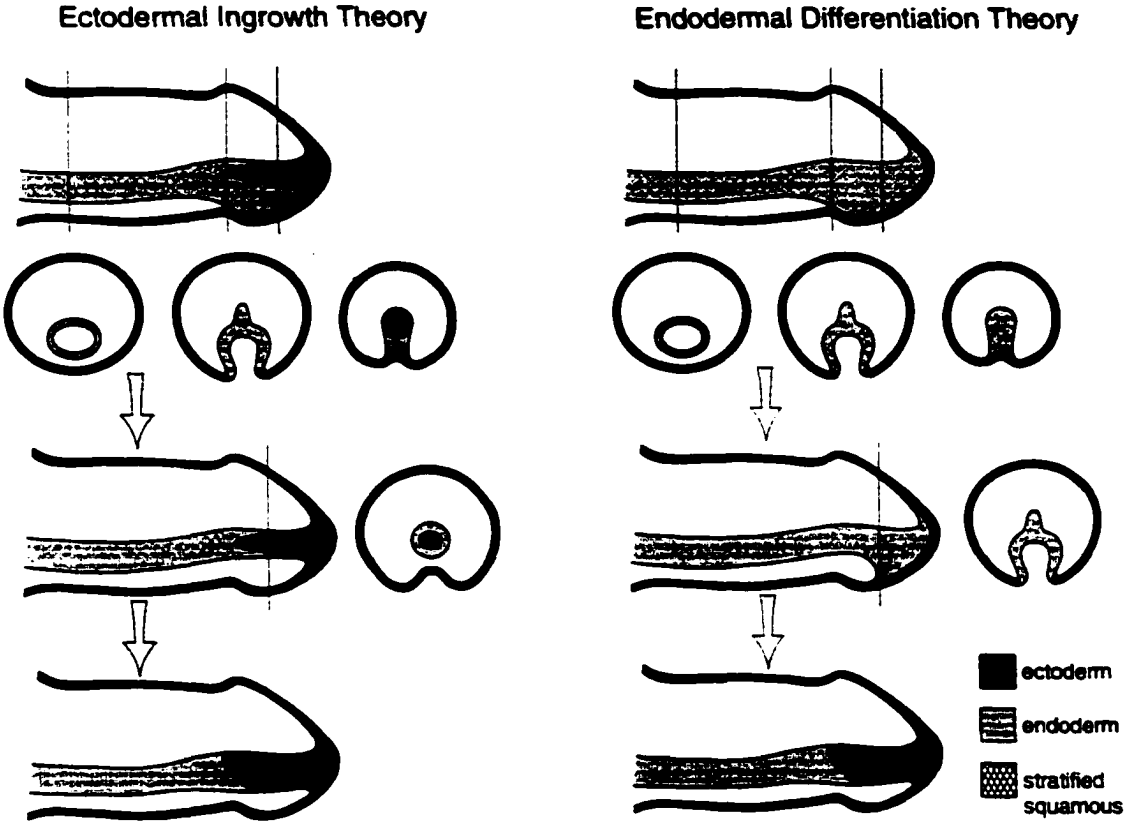

FIG. 2. Ontogeny of the male urethra. Authors of embryology textbooks have supported the theory that the glandar portion of the urethra was formed by the ingrowth of the epidermis. This theory presented the ontogeny of the glandar urethra as containing a mixture of both ectodermally and endodermally derived cells. In contrast to the conventional theory, a new possibility has been reported by Kurzrock, et al. (44). Findings from 36 human fetal phallic specimens from 5 to 22 weeks of gestation were sectioned and stained with cytokeratin-specific stains. The result of these experiments revealed that the urethral plate extended to the tip of the phallus throughout gestation. These results support a new theory of urethra ontogeny in the male.
Reprinted with permission from Kurzrock, E. A., L. S. Baskin, and G. R. Cunha, 1999.

ogens. Only recently, more information has been made available regarding the penile urethra as an immunocompetent site of the male urogenital tract (3, 73).

Epithelial Cell Biology

In order for most pathogens to be successful in the genital tract, they must first attach to the epithelial cell surface. While *Neisseria gonorrhoeae* and Ct infect the upper columnar epithelium, *Trichomonas vaginalis* infects the lower tract squamous epithelium. Epithelial cells represent an enormous amount of the total cellular human composition. Our external covering is composed of a thin multi-layer of epithelial cells, the outermost being keratinized. Internally, the most abundant type of epithelium is the mucosal epithelial tissue. There are four different types of epithelial cells: squamous, columnar, cuboidal, and transitional. As the body's barrier that is exposed to the external environment, each epithelial cell type has its own special function. They all act to form a boundary for the prevention of infection from any virus, bacterium, protozoan, and fungus and also possess the ability to prevent dehydration of body fluids. Besides percutaneous or traumatic entry of infectious organisms, most infections to humans occur at the epithelial cellular level. Therefore, a prime entrance site for the infectious agents or antigenic material is through the mucosal epithelium. Ct is a bacterial pathogen that can enter its hosts by sexual contact. Because Ct attaches to and grows in the epithelial cells, it evades innate and specific immune mechanisms that are operational in mucosal tissues.

Both the male and the female genital tract sites have stratified squamous and columnar epithelia. The male genital tract epithelium shares its embryological origin with the lower part of the female vagina. The high mitotic activity of the transformation zone be-

tween squamous and columnar epithelia of the penile urethra may create an unusually permissive site for Ct infection compared with other sites of the genital mucosa.

Chlamydia trachomatis *and mucosal immunology*

Ct's preferential site for growth, replication, and transfer to other hosts is found in the epithelial cells of the human mucosae. Ct's biology is unique and this genus has been identified as containing the only four bacterial species with such a distinctive developmental cycle. This developmental cycle allows a metabolically inert spore-like elementary body (EB) to enter into the host cell and replicate. By using the host cell machinery, Ct begins to increase in numbers of infectious components. When Ct has achieved its self-proliferative mission, the intracellular inclusion executes the cellular host by lysis, and the progeny are expelled into the lumen of the reproductive tract. This can occur in STD-associated infections of the reproductive tract for either sexes or other in mucosa-associated tissues such as the conjunctiva of the eye (trachoma) so that the newly released EBs may re-attach to a new host cell and begin the cycle once again (Fig. 3). Infection of epithelial cells may elicit humoral responses from both the systemic and mucosal immune systems. Ct infections also induce a CMI response that is important for keeping Ct-infections in check. From studies described in this dissertation, we know that Ct infections in the urethra of the male host stimulate production of increased amounts of Ig (IgA, IgG, and IgM), S-IgA, Ct-specific antibodies, and IL-8. The increased levels of S-IgA may help to prevent attachment of the EBs to the epithelial cells. IgA is known to bind to major outer membrane protein (MOMP) determinants on the EB surface. This binding may also prevent nutrient acquisition at intracellular sites (16). In the male urethra, we

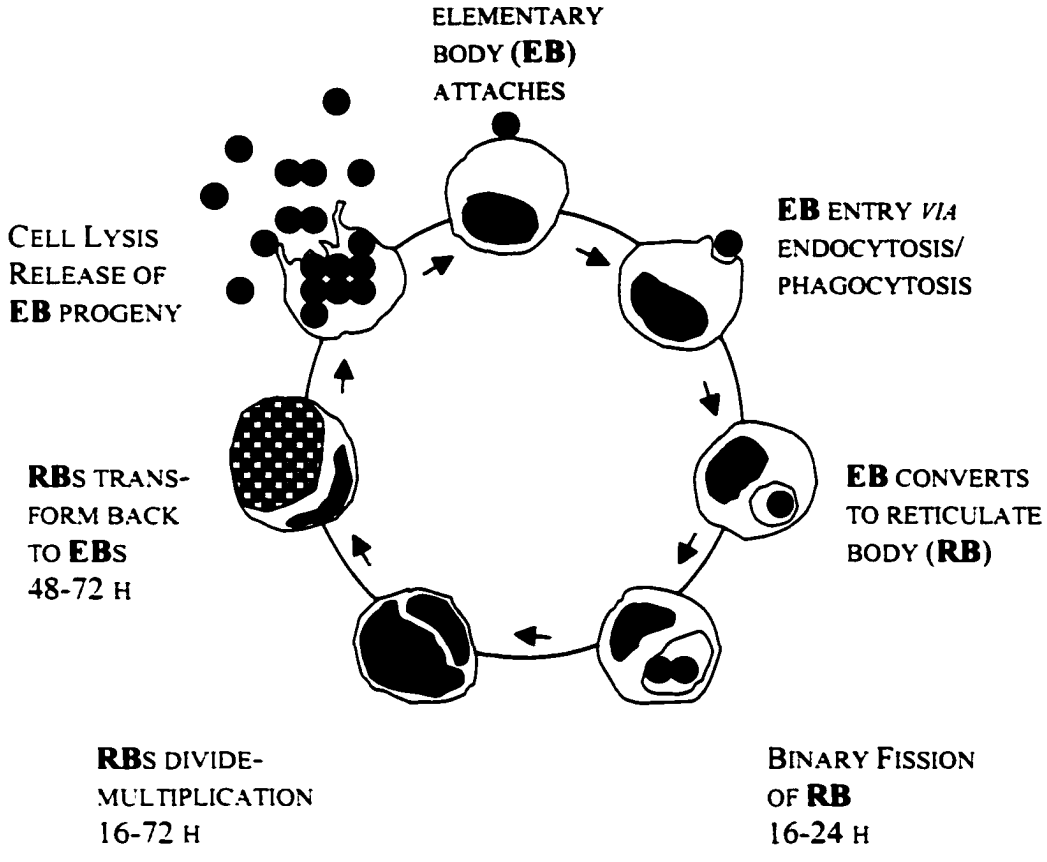

FIG. 3. The *Chlamydia trachomatis* developmental cycle. It begins with attachment of an inert elementary body (EB) to the cell surface. The EB is engulfed through active endocytosis or phagocytosis and enters into a trilaminar endocytic vesicle. It evades fusion with the phagosome and becomes a metabolically active reticulate body (RB). At this point, the RB actively reproduces through binary fission beginning at about 16-24 h. Within 16-72 h, the RB begins to fill the cytoplasmic space of the host cell. The metabolically active RBs condense, transforming back to inert EBs. The progeny of new EBs is now released by lysing the cell membrane and expulsion of the new EBs at about 48-72 h.

saw no increase in IFN-γ, but a possible trend may have been recognized, which is a deviation from results of animal models infected with Ct. However, if IFN-γ does play a part in Ct pathogenesis, it perhaps alters the biochemistry of the epithelium, preventing the Ct from acquiring all essential nutrients for growth. Both the presence of IgA and S-IgA, and the possible increase in IFN-γ levels at the site of infection may alter the host cell as well as the Ct metabolism in a manner that inhibits Ct growth and accelerates clearance of infection (16).

Persistent Ct infections result in tissue pathology. Scarring of the conjunctiva and fallopian tubes maybe a result of persistent infection. Persistence could possibly occur as a result of altered nutrient acquisition that allows for Ct growth but not in a replicative-competent state. Ct has the ability to create a long-lived sanctuary by inducing an anti-apoptosis mechanism. The mechanism inhibits mitochondrial cytochrome *c* release, thus preventing programmed cell death (16, 25). Due to the limited immune response at the mucosal sites of infection, and without intervention of antimicrobial therapy, Ct continues to grow and enlarge its areas of infected cells. Stealth or cryptic infections may occur in deeper tissues with prolonged Ct exposure that further exacerbates the innate and adaptive host defense resources (16).

Rationale and objectives for the present study

Immune responses to C. trachomatis *in the male urethra: rationale*

Extensively characterized local immune responses of the genital tract in Ct-infected animal models of either sex have provided evidence for a Th1-type immune response (69). An increased level of IFN-γ characterizes this response (84); few reports have suggested

that this also occurs in humans (reviewed in 16). Evaluation and detection of some components of the human immune system after Ct infection have been performed and reported, but are limited. The specimens of choice in all reported Ct studies were cervical samples in women and semen in men.

The first areas of the body that are encountered by the sexually transmitted Ct are the mucosal tissues of the female and male reproductive tracts. In the male (the host subject for the entirety of this report), the most likely anatomical site that is infected by any STD-associated organism is the penile shaft or the penile urethra. Since the targeted cells for Ct infection and proliferation are considered to be the columnar epithelial cells that line the male urethra and are not located on the exterior of the penile shaft, the logical area for determining the presence of immunoresponsive components would be the urethra of the affected male.

Until now there have been no reports of a thorough characterization of the local immune response to Ct in humans. There has been no comprehensive evaluation to detect (i) the presence of various cytokines at the site of local infection, (ii) the identity of cellular infiltrates, or (iii) the systemic and local Ig, in a way that gives an overview of the host's immune response to Ct.

Immune responses to C. trachomatis *in the male urethra: objective*

To characterize the mucosa-associated immune responses to Ct infections in males, our first objective was to determine the presence of eleven cytokines associated with cellular inflammation or with the Th1/Th2 cell subsets. To characterize the humoral response, our objective was to measure the levels of total Ig isotypes, the *Chlamydia-*

specific IgA and IgG, and to identify the types and numbers of cells present in the locally infected site. All of these determinations were compared with control subjects who, based on patient clinical records, were not infected with Ct, *Neisseria gonorrhoeae*, *Treponema pallidum*, or HIV in the genitourinary tract.

In vitro primary and commercial urethral epithelial cell cultures: rationale

Since the urethral epithelium is the target of many STD pathogens, a urethral cell culture system for *in vitro* evaluations is very important for evaluating STD pathogenesis. Because it would not be ethically acceptable to infect male subjects with Ct in the urethra (although another study has done this for the causative agent of gonorrhea [15]), a primary urethral cell-culture system would be the appropriate tool to address *in vitro* questions related to the epithelial cellular responses directed against Ct-infection that may be observed in the human male population. Only one similar primary urethral epithelial cell-culture system has been previously described in the literature (28, 35, 96). In the previously reported studies of primary urethral epithelial cells, the STD-associated organism that had been evaluated was *N. gonorrhoeae*. Two reasons have precluded many investigators from adopting similar STD pathogenesis studies in primary urethral epithelial cell culture: (i) the requirement of surgical explants to establish the primary urethral epithelial cell culture and (ii) the short life of the *in vitro* system due to cellular senescence.

Male urethral swab specimens that had been previously collected for diagnostic Ct culture (performed at University of Alabama at Birmingham's STD Program Laboratory) contained epithelial cells in the swab as well as in the transport medium. The fact that these specimens were readily accessible for evaluation made them ideal samples to try the

possibility to establish and sustain urethral epithelial cell growth. If these exfoliated cells could grow, they would provide necessary material to establish a primary cell culture system and determine whether the cells were of limited life span or possibly unlimited in number of population doublings.

In addition to establishing and evaluating a primary urethral epithelial cell culture system, a commercially available female carcinoma-associated urethral epithelial cell line (Hs769.T) was evaluated in parallel. Because there was no available information regarding these cells, characterization related to the cell line was generated in our studies.

In vitro primary urethral epithelial cell culture: objective

The main objective for establishing a primary male urethral epithelial cell culture was to provide an *in vitro* method for studying the pathogenesis of and innate immune responses (those contributed by the epithelium) to sexually transmitted disease (STD)-related microbial agents, specifically Ct. The second objective for initiating a primary cell culture system using easily available cells was to establish the conditions necessary to provide a tool that allows other investigators another *in vitro* cell culture evaluation resource.

In vitro C. trachomatis *urethral epithelium infection studies: rationale*

To mimic the *in vivo* responses produced by Ct-infected cells, without causing harm to the host, infection of urethral epithelial cells in an *in vitro* cell culture system has been needed. The advantage of such a system could be to provide more control of variables and ease of management. If urethral epithelia can be infected *in vitro* with Ct, then

the stage would be set for many Ct-infected cell-culture-associated studies to take place. The results of a successful Ct-infectable cell-culture system would perhaps allow investigators to answer many questions that have not been previously addressed due to limitations in other cell culture systems.

In vitro C. trachomatis *urethral epithelium infection studies: objective*

Goals of implementing an *in vitro* Ct-infected cell culture system would be to determine first whether the cells were infectable with Ct, and, second, what contributions the primary or established cell line of urethral epithelial cells have to the specific immune response. Other objectives that are not covered in this treatise could include: mechanism(s) of attachment for Ct EBs to urethral epithelia cells, high-density DNA array data for comparisons between Ct-infected and -uninfected cells, proteomics studies of Ct-infected cell-culture supernatants and cell lysates to identify changes in up-regulated expression or repression of genes, and the possible reconstitution of Ct-infected primary cell culture with autologous cellular immune components for evaluation of cellular interactions.

Relationship of the manuscripts in the dissertation. The following published chapter from the textbook *Mucosal Immunology* contains background information regarding Ct. The subsequent paper (in press) and the manuscripts in preparation present results of experiments that have been conducted to answer the questions related to the objectives discussed. The first journal article is presented in its original, article-length form, but is being published in Note form in the journal *Infection & Immunity*. The contents of this

article detail the male urethral mucosal immune responses to Ct as detected by appropriate laboratory assays for Ig, cytokines, cellular infiltrates, and antigen-specific antibodies.

The second article describes the growth of primary human urethral epithelial cells from penile urethral swabs. The growth of the swab-isolated epithelial cells and the markers used to identify the primary male urethral epithelial cells were compared to a female urethral carcinoma-associated cell line. These two types of cells are ultimately used in the third manuscript to determine the presence of cellular-derived cytokines when cells were infected with Ct and compared to uninfected controls.

MUCOSAL IMMUNOLOGY OF SEXUALLY TRANSMITTED DISEASES

by

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Mucosal Immunology, Second Edition, Chapter 93, pages 1468-1470

Chlamydia trachomatis

Chlamydiae are Gram-negative obligate intracellular bacteria that are incapable of producing adenosine triphosphate; thus they must obtain this and other nutrients from the host cell. Structurally, the outer cell wall of all *Chlamydia* species includes four major antigenic surface components: lipopolysaccharide (LPS), major outer membrane protein (MOMP) containing serovar-specific epitopes, and three heat shock proteins (HSPs), of 10 kDa, 57 kDa (part of the 60-kDa HSP family) and 70 kDa. Presently, the genus *Chlamydia* has four recognized species, *C. trachomatis, C. pneumoniae, C. psittaci,* and *C. pecorum* (which is not considered an important human pathogen). *C. trachomatis* is principally a human pathogen and is the causative agent of trachoma (the leading source of preventable blindness in the world), inclusion conjunctivitis, pneumonia of newborns, and genital infections. *C. trachomatis* primarily infects mucosal columnar and transitional epithelial cells of the eye and genitourinary tract. Acute genital infections are often asymptomatic in both men and women, usually involving the urethra in males, and urethra or endocervix in females. In about 10% of women, local ascension of the organism to the upper reproductive tract causes endometritis, salpingitis, and peritonitis (collectively referred to as pelvic inflammatory disease [PID]). In men, complicated ascending infections include epididymitis and prostatitis and are somewhat less common than upper genital tract infection in women. Other less common sites of primary sexually acquired mucosal infection include the oropharynx and rectum. Most of the long-term morbidity due to *C. trachomatis* occurs in women when upper tract infection causes scarring, which leads to chronic abdominal pain, infertility, and ectopic pregnancy. It is estimated that 50

million new cases of chlamydial infections occur globally a year (23), and approximately four million new cases occur in the United States (7).

Eighteen different *C. trachomatis* serovars have been identified. Trachoma (inclusion conjunctivitis) is caused by the trachoma biovar (serovars A, B, Ba, and C), which is hyperendemic in Asia, Africa, and South America. By contrast, most genital infections are caused by serovars D, Da, E-I, Ia, J, and K, which are common throughout the world, including North America and Europe. Another STD syndrome, lymphogranuloma venereum (LGV) is caused by serovars L_1, L_2, L_{2a}, and L_3, identified as the LGV biovar. LGV is associated with painfully inflamed inguinal lymph nodes (termed buboes). A mouse pneumonitis (MoPn) biovar also exists that has been used extensively in animal studies.

Genital infections are primarily localized to epithelium of the urethra, epididymis, and prostate in males, and the cervix, endometrium, and fallopian tubes in females. Extra -genital spread of infection is relatively uncommon, but Fitz-Hugh-Curtis syndrome, a PID-associated perihepatitis, may be seen in women (26) and Reiter's syndrome and sexually acquired reactive arthritis (SARA) are uncommon chronic complications of infection in either gender but occur most often in males.

Chlamydia exist in two forms over the course of a complex developmental cycle, living and dividing intracellularly as reticulate bodies (RBs), but being spread from person to person as metabolically inactive elementary bodies (EBs). The chlamydial growth cycle begins when attachment of infectious EBs results in entry into cytoplasmic vacuoles of host cells, which are able to avoid fusion with the epithelial cell lysosome. Intracellularly EBs undergo a metabolic and morphological change to become metabo-

lically active, noninfectious RBs, which propagate through binary fission, resulting in the formation of an intracellular inclusion at about 16 to 24 h following infection. After 48 to 72 h of replication and condensation, reorganization of the RBs leads to formation of EBs again. Rupture of the epithelial cell allows for the spread of infectious EB progeny to other cells to continue the life cycle. This replicative cycle, coupled with a sometimes ineffective host immune response, allows for a chronic progressive infection and the silent disease state of this organism.

There is a paucity of information regarding the local immune response to chlamydia in humans. However, animal studies using a variety of different animals (mice, guinea pigs, and primates), infecting strains, infectious inocula, and methods used to generate infection have provided some insights into such interactions. Most available information regarding the local immune response to genital chlamydial infection in humans has been obtained from studies of women, in whom the bulk of infections are diagnosed and from whom specimen collection is simpler. Local genital tract infection with *C. trachomatis* elicits both humoral and cell-mediated immune responses, each of which contributes to resolution of infection, although a predominant role has not been assigned for either.

In animal models (19) and to some degree in humans, untreated *C. trachomatis* infec-tions can resolve. In mice and guinea pigs, experimental *C. trachomatis* infection of the genital tract may be prolonged with immunosuppression, suggesting an important role for immune responses in controlling infection. A recently published, retrospective study of untreated chlamydial infections in humans also suggests that infection may be eliminated in humans without antimicrobial therapy. Parks et al. (1997) reported that of

74 patients with positive chlamydial cultures who did not receive recommended therapy at the time of initial screening, infection was no longer detectable in 21 (28%) at the time they returned for treatment. Resolution of infection without treatment was associated with increasing age and duration of infection, suggesting that host defenses may have contrib-uted to resolution (20).

Murine studies conducted using homozygous knockouts of genes encoding products critical to immune function indicate that both humoral and cellular factors play central roles in host response to *C. trachomatis* infections (16, 17, 28). In these studies, an intact cell-mediated immune (CMI) response alone could control and eliminate established infections, whereas antibody production was central to resistance to reinfection. Major histocompatibility complex (MHC) class I-restricted responses were necessary for both clearance of initial infections and resistance to secondary challenge with the same chla-mydial strains; intact MHC class II-restricted T-cell responses were required if animals were to clear primary genital infection. Gene deletions leading to deficient antibody production resulted in loss of the ability to resist reinfection following repeated genital inoculation of the same *Chlamydia* strains.

Numerous prior studies have demonstrated that humans with genital chlamydial infections produce antibodies to a number of chlamydial antigens; however, there has been little direct correlation of the serological response to the outcome of infection or resistance to reinfection (6, 16, 18). Previous studies have demonstrated that both locally produced secretory immunoglobulin A (SIgA) and serum-derived transudated immunoglobulin G (IgG) appear in the genital tract (11, 22). Locally produced, neutralizing IgA at mucosal surfaces is likely to play an important initial role in host response to genital

chlamydial infections (1). Potential target antigens such as LPS, MOMP, and heat-shock proteins (HSP) may stimulate mucosal plasma cells of the male and female reproductive tract to produce chlamydial antigen-specific SIgA. Brunham et al. (4) reported that *Chlamydia*-specific SIgA in human genital secretions correlates in a concentration-dependent fashion with the clearance of cervical infection. Barenfanger and MacDonald (1) showed that serovar-specific antibodies present in either local secretions or plasma immunoglobulin fractions from trachoma patients passively neutralize infectivity for the monkey eye, further helping to establish SIgA as a valuable component of the immune response.

Consistent with the concept of a common mucosal immune system, genital infection with *C. trachomatis* appears to induce specific antibody production at other sites. In a study by Haller et al. (10) about one-third of patients with chlamydial antigen-positive urethritis were found to have chlamydia-specific IgA in tears. No genital tract immunoglobulins were examined, and no antichlamydial IgG was found in tears of any of the urethritis-affected patients (10).

Although humans and experimental animals can become repeatedly infected, several lines of evidence suggest that in some instances at least partial protection from reinfec-tion develops. Indirect evidence supporting the development of protective immunity is the fact that naturally acquired chlamydial infections of the genital tract decrease with increasing age to a degree out of proportion with changes in sexual activity. Further evidence of protective immunity to chlamydial infection was described in a series of experimentally ocularly infected human volunteers by Jawetz et al. (14). In these studies, human volunteers were rechallenged with the same chlamydial serovar following resolu-tion of prolonged primary infection and were found to be resistant to reinfection.

However, when challenged with a heterologous serovar, they were susceptible, and more severe disease resulted. Similarly, Ward (29) described partial protection in trachoma vaccine trials that lasted just 1.5 to 3 years. Evaluation of a prototype vaccine for trachoma in humans and monkeys (8, 9) suggested that the humoral arm held some promise for protection against chlamydial infection; however, the response was serovar specific and short-lived. In addition, challenge with a heterologous chlamydial strain resulted in exacerbation, rather than protection from disease. Presumably, the heterologous challenge caused an enhanced cellular immune response characterized by increased production of interferon-gamma (IFN-γ) and other cytokines (see subsequent discussion), which in turn led to a delayed-type hypersensitivity-like response that actually resulted in a more severe disease. Such data have led to the concept that reinfection and delayed-type hypersensitivity immune responses cause the scarring in trachoma (29), tubal obstruction (21), and other chronic tissue damage, which characterize the sequelae of chlamydial infection.

Although antibody-mediated defense may prevent attachment of EBs and otherwise help to prevent infection, once inside the host cell, chlamydia EBs become inaccessible to and unaffected by the humoral immune response. Control of established infection ap-pears to require a cell-mediated immunological response necessitating activation of T cells, natural killer (NK) cells, or cytotoxic T-lymphocytes (CTLs), the activity of which is mediated in part through cytokine effectors.

Relatively little research has been done on the human cellular immune response to chlamydial infection. Thus current understanding of the role of cellular responses to chlamydial infections represents an extrapolation of animal studies. The cell-mediated

immune response of *Chlamydia*-specific T cells has been best studied in the murine models. Ramsey and Rank (24) found that adoptive transfer of phenotypically heterogenous T-cell lines from immune animals resolved genital tract infection in mice. These cells were derived from MoPn immunized mice and were stimulated *in vitro* before transfer to antibody-deficient recipients. The resolution of infection in these challenged mice exhibited kinetics similar to those in immunocompetent mice. Antichlamydial serum and secretory antibodies were not produced by these animals, yet they cleared the infection (24).

The relative role of different T-lymphocyte subpopulations in resolving chlamydial infection remains unclear, with experimental data available to support the role of both CD4+ and CD8+ lymphocytes. Both T-lymphocyte populations may contribute to con-trol of chlamydial infection through a common, cytokine-mediated pathway. The cyto-kines interleukin-1 and interleukin-6 are induced in response to chlamydial LPS in mu-rine pneumonia models infected with the Ct biovar MoPn, as well as in epithelial cell culture, and appear to contribute to resolution of infection (15, 25). Another important immune effector for host response in murine models is IFN-γ, which is produced from T cells in chlamydia-infected animals and which inhibits chlamydial growth *in vitro* in activated mononuclear phagocytes, fibroblasts, and epithelial cells (3). By inducing production of indoleamine 2,3-dioxygenase, IFN-γ may promote persistent chlamydial infection via tryptophan catabolism (2, 5).

Su and Caldwell (27) detected the production of a T-helper-1-associated cytokine response (interleukin-2, tumor necrosis factor-α, and IFN-γ) in T-cell populations harvested from mice with protective immunity to chlamydial infection after *in vitro* stim-

ulation. In addition, the cytokine interleukin-6, which is most often associated with T-helper-2, was detected as well (27). Thus, whereas both T-helper-1 and T-helper-2 cytokine profiles are detectable in these T-cell populations involved in protective immunity to experimental chlamydial infection, currently it appears that, at least in mouse pneumonitis models, the Th1 response is of somewhat more immediate importance for protective immunity and the resolution of experimental infections. Clearly, however, further investigation is needed to elucidate the natural history and significance of cell-mediated immunity in chlamydial infections.

Although recent studies have demonstrated their presence, the role of CTLs in host response to chlamydial infection requires further clarification. MHC class I-restricted CTLs are capable of lysing chlamydia-infected cells (12, 13), but it is not understood if the CTL response is protective or is related to the pathological consequences of infection.

In summary, then, although genital tract infections due to *C. trachomatis* are now the most common reportable STDs in the United States, very little is known about the natural history and host response to these infections. Rapidly accruing experimental animal studies suggest important roles for mucosal antibodies in prevention of infection, whereas a cellular immune response, possibly with a predominantly Th1 cytokine profile, appears to play a role in resolution of established infection.

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URETHRAL CYTOKINE AND IMMUNE RESPONSES IN *CHLAMYDIA TRACHOMATIS*-INFECTED MALES

by

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Infection & Immunity

ABSTRACT

Genital tract *Chlamydia trachomatis* (Ct) infections are a major health problem worldwide. The immune responses to Ct are poorly understood and have not been well evaluated in humans, especially in males. Penile urethral swabs, collected from Ct-infected (confirmed by polymerase chain reaction [PCR]) and uninfected males, were analyzed for cytokine, immunoglobulin, and antibody levels by enzyme-linked immunosorbant assay (ELISA). Local IL-8 levels were significantly higher in Ct-infected individuals than in Ct-uninfected males or non-Ct-non-gonococcal urethritis (NGU) males. Comparison of Ct-infected and Ct-uninfected males showed no significant differences among the levels of IL-1ß, IL-2, IL-6, IL-10, IL-18, or IFN-γ. IL-4, TGF-ß, and TNF-α were not detected in any urethral sample. Ct-specific IgA and IgG antibodies in urethral swabs were higher in the Ct-infected group than in the Ct-negative group. There was no difference in Ct-specific IgA and IgG antibodies in serum between Ct-infected and uninfected groups. These results indicate that the mild inflammatory response characteristic of Ct infection in males includes induction of low levels of Ct-specific antibodies of IgA and IgG isotypes, and of IL-8 possibly produced by epithelial cells and neutrophils at the infected site.

INTRODUCTION

Chlamydial infections affect more than 89 million people per year (12) globally. Ct causes blinding trachoma in much of the developing world and sexually transmitted diseases (STDs) worldwide. Chlamydial infections are the most common bacterial STD (8) in the United States, with approximately 4 million new cases per year.

Despite the critical role of both males and females in disease epidemiology, chlamydial infections are diagnosed far more often in females. Similarly, more information is available about the pathological aspects of the disease for females than for males. Contributors to this imbalance may be the greater morbidity associated with infection in females (7) as well as the relative ease of acquiring specimens from the female genital tract compared to obtaining specimens from the male tract. Better understanding of the host response of males is nonetheless important to efforts to unravel chlamydial pathogenesis and to develop chlamydial vaccines.

Chlamydia enter the host via mucosal surfaces that may exhibit their own cellular and humoral immune responses directed against infecting organisms. Mucosal antibody and cell-mediated immune (CMI) responses (30) have been described in experimentally Ct-infected animal models (guinea pig, mouse, and non-human primates) (17). Recent studies using Ct-infected mice have characterized the immunoregulatory response as Th1-type, reflecting production of IFN-γ and CMI, which are critical components of an effective immune response (18, 26).

Despite the plethora of information on immune responses to Ct in animal models, there exists little information regarding Ct-induced response(s) in humans. In the present study, we have evaluated human male urethral specimens for characteristic evidence of a Ct-immune response in a well-defined patient population.

MATERIALS AND METHODS

Patient population

One hundred and forty-two men, aged 13 to 46 years (median age 25), attending the Jefferson County Department of Health STD Clinic in Birmingham, Alabama, were included in this study. The population included 100 African Americans, 39 Caucasians, and 3 Hispanics. At the time of enrollment, each man had a penile urethral swab collected for preparation of a Gram-stained urethral smear and for *Neisseria gonorrhoeae* testing. A second swab was also collected and placed into sucrose phosphate (2-SP) transport medium for Ct culture (see Urethral specimen collection). The results of gonorrhea tests and chlamydial culture were obtained from the patient's record. HIV status was determined from the patient's record for 81 men. All specimens were subsequently tested by PCR (Roche Amplicor™ for *Chlamydia trachomatis*, Branchburg, New Jersey) performed on chlamydial transport medium to classify the patients as negative or positive for chlamydial infection. The total enrollment was divided into three groups. The Ct-infected group (n = 71) was defined as PCR-positive for Ct only and negative for *Treponema pallidum* and *N. gonorrhoeae*. A second group (n = 15) containing patients with non-gonococcal urethritis (NGU) who were Ct-PCR negative was identified as non-Ct-NGU. Non-Ct-NGU was defined by symptoms and a urethral smear. The third group was considered uninfected controls (n = 56) who were not infected with Ct, *N. gonorrhoeae*, or *T. pallidum* (by laboratory methods), or with HIV (if available from the patient's record). Due to the limited amount of specimen available for assay, no one sample was analyzed for all components presented in this report.

The University of Alabama at Birmingham's Institutional Review Board and the Quality Improvement Office of the Jefferson County Department of Health approved the study.

Patient clinical status

Patients were classified as symptomatic (dysuria with or without urethral discharge) or asymptomatic based on patient complaints and clinical findings. A Gram-stained urethral smear was examined for the presence of intracellular diplococci (presumptive for *N. gonorrhoeae*) and for the presence of polymorphonuclear leukocytes (PMNs). The number of PMNs observed on the Gram-stained smear was scored at a cut-off value of $\geq$ 5 PMN/Oil Immersion Field (OIF) from the average of five fields. The patients' clinical status, Ct-PCR results, and Gram-stain interpretation are presented in Table 1.

Urethral specimen collection

Dacron-tipped stainless-steel shaft swabs were used to sample the penile *fossa navicularis* and anterior urethra. The urethral swab was inserted and withdrawn with a rotary motion and placed into a glass vial (Wheaton Glass, Wheaton, Ill.) containing 1.5 ml of 2-SP transport medium (0.2M sucrose, 0.07M potassium phosphate monobasic, 0.1M potassium phosphate dibasic, fetal bovine serum [2%], 12.5 units/ml nystatin, 5 mg/ml gentamicin, and 12.5 mg/ml vancomycin). Swab shafts were cut and discarded allowing the swab tip to remain in the transport vial.

TABLE 1. Urethral Gram-stain smear and PCR results in symptomatic and asymptomatic males with and without Ct infection

Patient Group[a]	Clinical Status[b]	Number	Urethral Gram-stain smear[c]		
			No PMNs	1-4 PMNs	≥ 5 PMNs
Uninfected (Control)	Symptomatic	4	3	1	0
	Asymptomatic	51[d]	44	6	1
CT-negative (Non-Ct-NGU)	Symptomatic	10	0	1	9
	Asymptomatic	5	0	0	5
Ct-positive (Experimental)	Symptomatic	50	6	1	43
	Asymptomatic	21	9	4	8

[a] As defined by PCR result.
[b] Defined by the presence of a urethral discharge and/or dysuria.
[c] Number of PMN per OIF.
[d] One patient was asymptomatic and PCR-negative but did not have a Gram-stain smear performed.

Ct cell culture

Diethylaminoethyl (DEAE)-dextran-treated McCoy mouse fibroblasts were inoculated with 100 µl of the 2-SP transport medium in duplicate wells of a 96-well plate, and another 100 µl were placed into a third well on a second plate (28). The two plates were incubated for 48-72 h at 37°C. Both wells of the first plate were stained with fluorscein-isothyocyanate-tagged monoclonal antibodies specific for *Chlamydia*. One stain's target was the Ct major outer membrane protein (MOMP) (Syva Microtrak, Palo Alto, Calif.) and the other stain's target was the chlamydial lipopolysaccharide (LPS) (Kallested, Diagnostics Pasteur, Chaska, Minn.). Inclusions were observed in infected cultured fi-

broblasts by using a fluorescence microscope (Axiovert, Zeiss, New York, N.Y.), and positive cultures were graded on a 1+ to 4+ scale based on the number of elementary bodies or inclusion-forming units observed following 48-72 h incubation. If the first plate was negative for Ct, the corresponding well from the second plate was sub-cultured to a second fibroblast monolayer cell-culture well, incubated for a further 48-72 h, and then stained with the LPS monoclonal antibody.

PCR procedure

The evaluated population was selected from patients cultured for Ct. PCR, a more sensitive test than culture (35), was performed on all specimens to further reduce the probability of low-level positivity not detected by Ct-culture. Ct-PCR detection was performed using a commercially available method (Roche) adapted to detect the presence of Ct within the transport medium as described (28). A 100-µl volume of 2-SP transport medium was added to 900 µl of Roche swab liquid transport medium, and an aliquot of the mixture was further mixed with an equal volume of Roche swab diluent. The detection of Ct was then made according to the manufacturer's instructions.

Cytokine ELISA assays

Cytokine concentrations (except IL-18) in transport medium were assayed by sequential ELISA (34) starting at an initial dilution of 1:2. Serum cytokines were assayed by conventional ELISA. Ninety-six-well plates (Dynatech Labs, Chantilly, Va.) were coated with anti-human antibodies to IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12 (p70), IL-18, TGF-β, or TNF-α (R&D Systems, Minneapolis, Minn.) and incubated overnight.

ELISAs for the detection of IL-4 and IFN-γ (Phar-Mingen, San Diego, Calif.) were performed following the manufacturer's instructions.

For the cytokine assays, the 2-SP specimen swabs were vortexed for 30 s, and the initial 125-μl aliquot of the dispersed swab fluid was placed into the first set of coated wells in duplicate, then serially two-fold diluted and incubated at room temperature overnight. Respective suppliers' internal standards were treated in a similar manner. The contents of each well were then sequentially transferred from one cytokine capture-detection plate to another in volumes decreasing by 20 μl in each step to the lowest volume of 65 μl, providing four transfers per specimen. The captured cytokines were detected with corresponding mouse anti-human antibodies followed by peroxidase-conjugated anti-mouse IgG (Southern Biotechnology Associates, Birmingham, Ala.) and incubated for 4 h at room temperature. A substrate containing *o*-phenylenediamine (OPD) and H_2O_2 in citrate-phosphate buffer (pH 5.0) was used for the color development reaction, which was stopped (0.1M sulfuric acid) after 15 min. Plates were read at 490 nm on a Vmax plate reader (Molecular Devices Inc., Hanover, N.J.) or a Bio-Tek EL312e plate reader (Bio-Tek Instruments, Winooski, Vt.) interfaced to a Macintosh computer for data storage and retrieval. For each assay plate generated, data were interpolated on a standard curve with a statistical evaluation package using 4-parameter logistic algorithms (DeltaSoft 3, Biometallics Inc., Princeton, N.J.).

Total IgG, IgM, and IgA ELISA

Total levels of IgG, IgM, and IgA were measured by ELISA using appropriate anti-human IgG, IgM (Dako, Carpinteria, Calif.), and IgA (Jackson ImmunoResearch,

West Grove, Penn.) capture antibodies placed into 96-well plates and incubated overnight at room temperature. The same method of sequential specimen transfer was used for the IgG and IgM assays in swab specimens, but not for serum samples. Standards and specimens were placed on the respective plates in duplicate and allowed to incubate overnight. Appropriate standard curves were generated for each Ig isotype using serial dilutions of pooled human serum with known Ig concentrations (IgG and IgM: Moni-Trol E, Baxter, McGraw Park, Ill.; IgA: The Binding Site, Birmingham, England). Bound IgG or IgM antibodies were detected with peroxidase-conjugated anti-human IgG or IgM (Dako) after 4-h incubation and read as described above. IgA was detected after 3-h incubation with biotinylated anti-human IgA (Bio-source International Inc., Camarillo, Calif.). Extr-Avidin peroxidase (Sigma, St. Louis, Mo.) was added for a 1-h incubation. Color was developed with the OPD substrate and read as previously described.

Secretory IgA (S-IgA), IgA1, and IgA2 ELISA

Plates were coated with monoclonal anti-human SC (Sigma), anti-human IgA1 or IgA2 antibodies (Accurate Chemical and Scientific Corp., San Diego Calif.) overnight at room temperature. Next, duplicate serial two-fold dilutions of swab specimen were incubated overnight at room temperature followed by a wash and 3-h incubation with biotinylated anti-human IgA (Biosource International Inc., Camarillo, Calif.). The standards, S-IgA purified from colostrum (24), IgA1 and IgA2 (The Binding Site, Birmingham, England) were treated in a similar manner. ExtrAvidin peroxidase (Sigma, St. Louis, Mo.) was added for a 1-h incubation. The ELISA development and plate reading were performed as previously described.

Antigen-specific antibody detection

A synthetic peptide associated with the Ct MOMP variable domain IV was used to detect the presence of antigen-specific IgA and IgG antibodies in ELISA (27) (Labsystems, Helsinki, Finland), according to the manufacturer's instructions. Although this ELISA was designed for use with serum specimens, Ct-specific antibodies were detected in the swab extracts by a modified procedure in which an aliquot of the transport medium was mixed with an equal volume of assay diluent. This mixture was incubated on the peptide-coated plate for 30 min at 37°C. Following a wash step, horseradish peroxidase-conjugated anti-human IgA or anti-human IgG was added and incubated again for 30 min at 37°C. The sample was washed again and a substrate solution containing tetramethyl benzidine/H_2O_2 was added and incubated for 15 min at room temperature in the dark. The reaction was stopped after 15 min then read at 450 nm.

Secretory leukocyte protease inhibitor (SLPI) ELISA

Detection of human SLPI was performed according to manufacturer's directions (Quantikine, R&D Systems).

Cytospin cell stain and differential count

Cellular components from urethral specimens less than 24-h old in 2-SP were concentrated by cytospin centrifugation onto glass slides. The slides were allowed to air-dry, and were fixed and stained with a modified Wright-Giemsa stain (Diff-Quik, Baxter Healthcare, Chicago, Ill.), air-dried, and mounted in Permount (Difco Labs, Detroit, Mich.). Differential cell counts were made using a brightfield microscope (Zeiss) and a

100× oil-immersion objective. Results are given as the percentage of 100 leukocytes counted or of the total number of leukocytes present if < 100.

Statistical methods

Comparisons of the medians for all results were evaluated by the Mann-Whitney *U* test, using InStat for Macintosh computers (Graphpad Software, Inc. San Diego, Calif.). Differences for all comparisons were considered significant at $P < 0.05$.

RESULTS

Cytokine levels in the male urethra

We examined swab specimens in transport medium for the presence of cytokines in the control group (n = 56), non-Ct-NGU group (n = 15), and the Ct-positive-NGU group (n = 71) (Table 2). Of the cytokines evaluated, only IL-8 was significantly increased ($P < 0.0001$) in Ct-PCR-positive subjects when compared with Ct-PCR-negative subjects (Fig. 1). Levels of IL-8 were similar in non-Ct-NGU subjects and the control subject group ($P = 0.500$). Levels of inflammatory cytokines IL-1β and IL-6 were not appreciably higher in the urethral swab fluids of Ct-infected males than in the fluids of uninfected males. Furthermore, levels of the Th1-associated cytokines, IL-2, IFN-γ, and IL-18, as well as Th2 cytokines, IL-4, IL-6, and IL-10, and the inflammatory cytokine IL-1β in the urethral swab fluid of Ct-infected males did not differ significantly from those in the control group (Table 2). IL-4, TGF-ß, and TNF-α were below the detection limits (set at 30 pg/ml) for this method in all specimens. Levels of IL-18 were detectable in the

TABLE 2. Cytokine levels in urethral swab specimens

Patient groups	Cytokine level Mean ± SEM (n)						
	IL-1β pg/ml	IL-2 pg/ml	IL-6 pg/ml	IL-10 pg/ml	IL-12 (p70) pg/ml	IL-18 ng/ml	IFN-γ pg/ml
Uninfected[a]	26.0 ± 4.8 (13)	57.5 ± 20.6 (15)	152 ± 37.4 (4)	6.6 ± 1.3 (12)	354 ± 139 (11)	5.8 ± 0.5 (37)	0.6 ± 0.1 (10)
Non-Ct-NGU[b]	42.6 ± 9.7 (7)	69.6 ± 26.0 (7)	Not Done	7.0 ± 0.69 (7)	392 ± 160 (6)	Not Done	0.7 ± 0.1 (7)
Ct-Infected[a, b]	49.1 ± 13.8 (23)	50.3 ± 11.6 (19)	94.3 ± 14.6 (6)	16.0 ± 7.7 (18)	140 ± 43.1 (16)	7.6 ± 0.9 (40)	1.6 ± 0.6 (24)
P^a	0.73	0.72	0.17	0.98	0.21	0.32	0.092
P^b	0.13	0.53	NA	0.34	0.47	NA	0.360

Differences for all comparisons were considered significant at $P < 0.05$.

[a] P value determined by comparison of uninfected to Ct-infected males (Mann-Whitney U test).

[b] P value determined by comparison of males with non-Ct-NGU to Ct-infected males (Mann-Whitney U test).

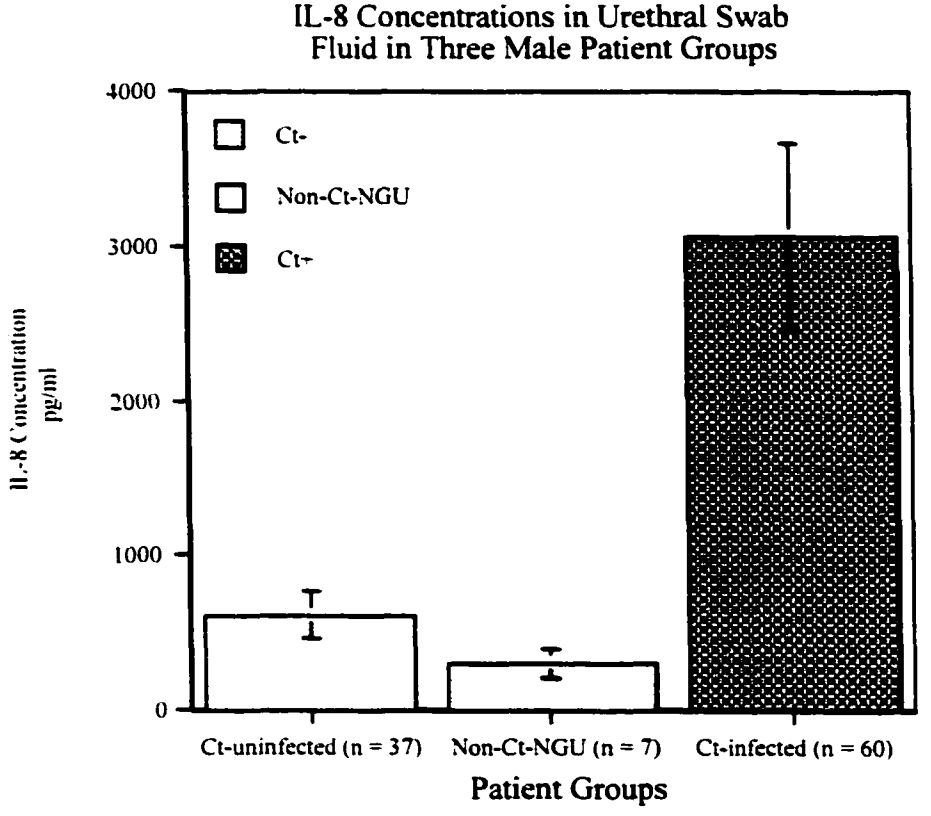

FIG. 1. Comparison of IL-8 levels in Non-Ct-NGU, Ct-uninfected, and Ct-infected males. The bar graph represents the mean level of IL-8 for each group. Significant differences were found in IL-8 levels in males infected with *C. trachomatis* compared to those who were not infected. Error bars represent SEM. ($P < 0.0001$).

ng/ml range in the Ct-infected and Ct-uninfected groups with little variance and no statistical difference between the groups ($P = 0.320$). The non-Ct-NGU group was not evaluated for IL-18 levels.

Cytokine levels in serum of Ct-infected and -uninfected males

Serum specimens from 9 Ct-infected and 17 Ct-uninfected males were evaluated for the same battery of cytokines tested in the urethral swab medium (except for IL-18). No statistically significant differences were found between the serum cytokine levels of the two groups except for IL-8; IL-8 levels were dramatically lower in Ct-infected than in the Ct-uninfected group ($P = 0.0016$, Table 2).

Ig levels in the urethral swab fluids of Ct-infected and -uninfected males (Table 3)

Total IgA, IgA1, IgA2, IgG, and IgM levels were higher in the urethral swab fluids from Ct-infected males than in swab samples from the Ct-uninfected males (for all Igs, $P < 0.05$); a significant increase in the levels of S-IgA in the urethral swab fluids was observed in the Ct-infected group when compared with the uninfected group ($P = 0.0071$). Levels of IgA1, IgA2, and S-IgA were not determined for non-Ct-NGU patients.

Ig levels in the serum of Ct-infected and -uninfected males

Total levels of serum IgA, IgG, and IgM were not significantly elevated in the Ct-infected group when com-pared with the uninfected group (data not shown; for all Ig $P >$

TABLE 3. Total Ig concentrations in urethral swab specimens

Patient groups	[Ig] ng/ml, mean ± SEM (N)					
	IgA	IgA1	IgA2	S-IgA	IgG	IgM
Uninfected[a]	898 ± 245 (15)	582 ± 178 (15)	252 ± 64 (15)	1046 ± 349 (15)	1113 ± 247 (13)	53 ± 15 (12)
Non-Ct-NGU[b]	3329 ± 810 (7)	Not Done	Not Done	Not Done	4310 ± 2061 (7)	133 ± 64 (7)
Ct-Infected[a, b]	1503 ± 244 (18)	846 ± 121 (17)	994 ± 190 (17)	2761 ± 507 (18)	24007 ± 12183 (28)	394 ± 104 (28)
P^a	0.0350	0.0450	0.0140	0.0071	<0.0001	0.0001
P^b	0.0020	NA	NA	NA	0.0150	0.2600

[a] *P* value determined by comparison of uninfected to Ct-infected males (Mann-Whitney *U* test).

[b] *P* value determined by comparison of males with non-Ct-NGU to Ct-infected males (Mann-Whitney *U* test).

0.02). There were no differences in serum Ig levels between the non-Ct-NGU subjects and the Ct-uninfected subjects (data not shown; for all Ig $P > 0.70$).

Antibody response to Ct

The levels of Ct-specific IgA and IgG in urethral swab transport medium were higher in Ct-infected compared to Ct-uninfected males ($P < 0.0001$, Table 4). Serum from 9 Ct-infected individuals and 11 uninfected individuals (non-Ct-NGU subjects were not included) revealed no differences in antibody levels ($P > 0.5$, Table 4).

The commercial assay for detection of Ct-specific antibodies in serum had established cutoff for positive, negative, and equivocal values criteria of both IgA and IgG. According to this, only 4 of 9 Ct-positive subjects had Ct-specific serum IgG antibodies, and 5 were considered negative; only 2 of 9 Ct-positive subjects had serum IgA antibodies. In Ct-negative subjects, 2 of 11 males were considered positive for Ct- specific IgG serum antibodies, and only 1 of 11 was positive for serum IgA Ct-specific antibody.

Differential urethral swab cell counts

Differential cell counts of lymphocytes, monocytes, and PMN from swab specimen samples for the three groups are shown in Table 5. The numbers of monocytes in both the Ct-infected and the non-Ct-NGU men were lower than those in the uninfected men ($P = 0.0381$). The total numbers of lymphocytes were equal among the groups. When compared to the PMNs in the uninfected group, the numbers of PMNs were higher in the non-Ct-NGU group, but not in the Ct-infected group. Six asymptomatic males (5

TABLE 4. Ct-specific antibodies in urethral swab specimens and serum

Patient group	CT-specific antibody of isotype: Absorbance units, mean ± SEM (N)			
	Urethral IgA	Urethral IgG	Serum IgA	Serum IgG
Uninfected	0.144 ± 0.03	0.076 ± 0.004	0.577 ± 0.252	0.852 ± 0.298
	(30)	(33)	(11)	(9)
Ct-infected	0.548 ± 0.129	0.187 ± 0.047	0.852 ± 0.298	1.031 ± 0.294
	(33)	(33)	(11)	(9)
P[a]	< 0.0001	< 0.001	0.66	0.50

[a] *P* value determined by comparison of uninfected to Ct-infected males (Mann-Whitney *U* test).

TABLE 5. Leukocyte cell type numbers in urethral swab specimens

Patient group	N	Differential cell count (% of total) Mean ± SEM		
		Lymphocytes	Mononuclear cells	PMNs
Uninfected[a]	9	5.9 ± 2.1	70.2 ± 7.1	23.9 ±7.2
Non-Ct-NGU[b]	8	5.0 ± 1.5	39.0 ± 10.1	45.3 ± 7.1
Ct-Infected[a, b]	11	7.8 ± 1.7	45.3 ± 7.1	44.3 ± 8.1
P^a		0.50	0.038	0.13
P^b		0.96	0.036	0.036

[a] *P* value determined by comparison of uninfected to Ct-infected males (Mann-Whitney *U* test).
[b] *P* value determined by comparison of males with non-Ct-NGU to Ct-infected males (Mann-Whitney*U* test).

non-Ct-NGU and 1 Ct-positive, refer to Table 1) had no leukocytes observed on the cyto-centrifuged slide specimens.

SLPI levels in urethral swab specimens

SLPI was found in all specimens (Fig. 2), and all three groups had comparable levels. These findings agree with the similar total numbers of PMN in both the Ct-infected and -uninfected males ($P = 0.1308$).

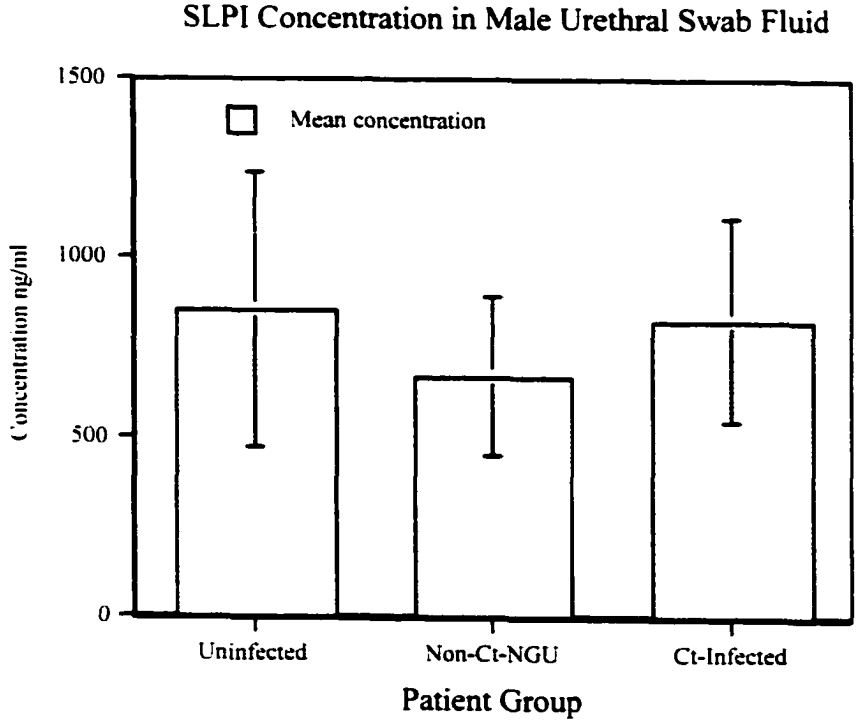

FIG. 2. SLPI presence in male urethral swab fluid. SLPI levels were detectable in urethral swab specimens from uninfected males, non-Ct-NGU males, and those infected with Ct. The levels did not vary among the groups, indicating that Ct-infection does not up-regulate secretion of SLPI into the urethra Error bars represent SEM.

Ct-culture grading and comparison with cytokines and Ig

There was no correlation between the number of Ct inclusion-forming units in culture and the levels of IL-8, other cytokines, or Ig from these same specimens.

DISCUSSION

To characterize the immune responses to Ct in infected males, we evaluated concentrations of cytokines, Ig, and cells collected with the same male urethral swabs used to culture the organism. To our knowledge, this study provides the first comprehensive survey of the local response associated with human chlamydial infection. We detected higher levels of IL-8, total Ig, and Ct-specific antibodies at the locally affected among infected participants than among the uninfected control group.

However, our findings differ from those of investigators using animal models of chlamydial infection. While immune responses to Ct in animal models seem to reflect a typical Th1-type response to initial and repeat infection (26), the present study reports only a mild cellular inflammatory response directly associated with Ct.

In some previous studies, semen was used to determine the presence of Ct and Ct-specific antibody levels (20, 21, 32). However, this fluid is predominantly derived from sources other than the urethra and, thus, may not reflect the primary site of infection (29). The results from the present study probably portray the local responses to Ct more accurately than can be achieved from evaluations of other body fluids such as urine and semen, which only pass transiently through the site of infection, the male urethra.

Because Ct is an ascending infection, sampling upper regions of the urethra could generate different results, but this type of sampling would be more invasive. The columnar epithelial cells that are primary targets for Ct are located further up the urethra and would therefore be less accessible to swab sampling. In this study, the swab sampling collected only the distal urethral squamous epithelium and associated fluids most likely derived from the intra-epithelial glands of Littre'. These fluids contain S-IgA and secretory-IgM, which are continuously secreted into the lumen to serve as a protective barrier for the distal urethra (3). A break in the squamous epithelium, possibly due to swab trauma, may help to increase the amount of fluids and Ig in the sampled areas by increasing transudation of the plasma across the epithelium.

Of the eleven cytokines assayed, only IL-8 levels were elevated in Ct-infected men when compared with the uninfected groups; however, these high levels of IL-8 were not associated with significantly elevated numbers of neutrophils in the swab specimens from these subjects. Despite an increased number of PMNs in the non-Ct-NGU group, the IL-8 levels for this same group were comparable to IL-8 levels detected in the Ct-negative group. IL-8 is a CXC chemokine that is produced by epithelial cells, monocytes, and neutrophils, and appears to mediate the recruitment of neutrophils to areas of inflammation or infection (4, 14). Primary endocervical epithelial cells as well as cell lines (HeLa, SiHa, and HT-29) shown by Rasmussen et al. (31), are capable of secreting IL-8 when infected with Ct *in vitro*. Hang et al. (13) have detected IL-8 by cytokine staining in urethral epithelial tissue from both disease-free and diseased subjects. These results suggest that epithelial cells lining the urethra not only produce IL-8 normally, but also, when infected with Ct, produce it at increased levels. From males diagnosed with NGU

for the first time, urethral exudates are reported to show predominantly PMNs compared to urethral exudates from males who experience a subsequent episode of NGU (33). Neutrophil recruitment is most likely the result of IL-8 production by mucosal epithelial cells (2) and by the neutrophils themselves, which are among the primary responders to NGU caused by Ct. It has been reported that neutrophils alone play a critical role in the control of the early stages of Ct infection in an animal model (5).

The cytokine IL-18 has been implicated as important in the regulation of both innate immunity and acquired immune responses (22). Lu et al. (19) have shown that cell lines infected with Ct do in fact produce the active form of IL-18 after cleaving its pro-form with caspase-1. However, in the present study, there was no statistically significant difference in the levels of IL-18 or any other cytokine (apart from IL-8) between Ct-infected and uninfected males.

SLPI is present in many human secretions including tears, nasal secretions, cervical mucus, and seminal fluid (1, 9, 11, 25), but it has never been described in the male urethral fluid. As a consequence of PMN activity associated with Ct-infections, we expected that neutrophil elastase would be increased, as reported previously in Ct-proven male urethral infections or urethritis (10). Elastase can damage the epithelium and possibly interfere with host defenses (36). Because SLPI acts as a major inhibitor of neutrophil elastase in secretions (6), has microbicidal activity (16), and is stable in an acidic environment (11), we evaluated urethral specimens for levels of SLPI in Ct-infected individuals and compared these specimens to the specimens of the other two groups. However, no increases in this protein were measured in the Ct-infected patients.

The source of the increased levels of Igs in the Ct-infected male urethra may be local production, or transudation of plasma proteins into the genital tract secretions. The increase of Ig may have been a response to possible trauma and inflammation as similarly discussed in relation to seminal fluid (23). The levels of S-IgA were significantly higher ($P = 0.0071$) in Ct-infected than in the uninfected males. As a major characteristic of the mucosal immune system, S-IgA levels most likely represented locally produced IgA. Elevated Ct-specific IgA antibodies were found in swab specimens from Ct-infected males, but not in serum. Higher levels of Ct-specific IgG antibodies were found in swab specimens from the Ct-infected group than in those from the uninfected group; however, no increased Ct-specific IgG was detected in serum. Ludwig et al. (20) found Ct-specific IgA and IgG antibody levels in seminal fluid (20). However, as in the present study, they found no correlation between Ct-disease and systemic anti-Ct-antibodies (20).

The results of this study indicate that Ct infection of the male urogenital tract induces minimal immune and cytokine responses against the infecting organism. Similarly, minor antibody responses in cervical mucus from women infected with *N. gonorrhoeae* have been reported by Hedges *et al.* (15). Thus, immune responses in the urogenital tract to sexually acquired pathogens appear to be limited in magnitude. Although low, compared to many other infections, the levels of Ct-specific antibodies were higher in the urogenital tract than those found in the systemic compartment.

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CHARACTERIZATION AND COMPARISON OF PRIMARY MALE URETHRAL EPITHELIAL CELL CULTURE TO A HUMAN URETHRAL EPITHELIAL CELL LINE

by

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ABSTRACT

The study of sexually transmitted disease (STD) pathogenesis in males has been limited due to the lack of primary epithelial cell culture models derived from normal human genitalia or the lack of appropriate cell lines for evaluation. The present study evaluated the ability to grow primary urethral epithelial cells from readily available swabs. Approximately 9.3% (29/311) of all urethral swab specimens evaluated were capable of generating an ongoing primary cell culture. Comparisons were made between a commercially available female urethral carcinoma epithelial cell line and the primary cells for growth kinetics, cytokeratin staining, and the ability to be infected with Ct. The results of these experiments provide an *in vitro* system for studying pathogenesis of Ct infection in both a urethral cell line and in a primary cell culture system obtained from male urethral epithelia.

INTRODUCTION

Most STD pathogens, including bacteria, viruses and the parasitic protozoan *Trichomonas vaginalis* enter into the host via mucosal surfaces (4). In both males and females, the mode of infective transmission involves intimate contact with the mucosal epithelial surfaces (14, 15). Since the epithelial surfaces are the initial sites of many infectious processes, the host epithelial barrier has provided a defense against pathogenic intrusion. The epithelium provides a physical barrier between the host and the external environment. Epithelial cells are short-lived and easily sloughed, carrying with them many potential invaders. The mucosal epithelia are exposed to innate immune factors including mucins (2, 6, 7), human defensin 5 (12), and secretory leukocyte protease inhibitor, all of

which help prevent infection (1). Secretions of mucosal tissues contain specific humoral factors such as immunoglobulins to combat any organisms that may breach the physical barrier. Therefore, defensive mechanisms provided by the epithelium itself and its associated lymphoid cells are involved in defenses against infective pathogens. Although many of these innate and specific immune factors have been described in the female reproductive tract, information about the humoral immune factors in the male genital tract is lacking. Although a report by Pudney and Anderson (11) has demonstrated the presence of cellular and humoral immunological components in the male urethra, few reports of immune system-directed responses against STD-related urogenital pathogens are available.

The external male genitalia, as well as the penile urethra are sites of primary infection following STD exposure. Although a few STD-related organisms infect the external penile shaft (e.g., herpes, HPV, and chanchroid caused by *Haemophilus ducreyi*), the majority of STD-associated infecting organisms target the penile urethra for entry into the host (1). Most infections with syphilis, *Neisseria gonorrhoeae*, Hepatitis B virus, HPV, and *Chlamydia trachomatis* (Ct) primarily involve the urethral cells. As a first line of defense against invading pathogens, the epithelium may have developed methods that promote deleterious consequences to the infecting organisms.

The epithelial cells that line the urethra from the distal external os or *fossa terminalis* to the proximal prostate urethra and bladder vary in their morphology and function. The external urethral opening, located in the glans penis is considered a part of the glandar tissue, which is lined with non-keratinized, stratified squamous epithelia of endodermal origin (5). Similar epithelia line the *fossa navicularis* until the beginning of the penile urethra, where the stratified squamous epithelia becomes pseudo-stratified columnar epi-

thelia. The ascending epithelium changes to stratified columnar epithelium, which lines the remainder of the urethra until it is in proximity of the prostate, where the epithelium becomes more transitional and is identified as prostatic urethra (1).

Attempts to study the relationship between the host tissue and invading pathogens have been hampered by limited cell culture techniques that utilize either a commercially available female urethral cell line or a primary urethral cell culture system. Therefore, we evaluated the potential for the *in vitro* growth of primary urethral epithelial cells from the male genital tract. Male urethral swab specimens that were collected during a routine screening procedure for detection of Ct were used. These specimen swabs were used to inoculate a Ct diagnostic cell culture system. After we used the swabs for Ct-culture, we were able to initiate and maintain primary urethral epithelial cell cultures from several male subjects. The present study reports the methods used and the characterization of these cells using transmission electron microscopy, immunohistochemistry, and western blotting to determine cell products. A commercially available female urethral cell line that was not previously characterized was evaluated in parallel. Furthermore, both the primary cells and the cell line were infectable with the STD pathogen Ct.

MATERIALS AND METHODS

Patient population

A total of 311 men attending the Jefferson County Department of Health STD Clinic in Birmingham, Alabama, were included in this study. The ages of the men ranged from 16 to 84 (median age 27 years). A total of 279 African Americans, 27 Caucasians, 3 Hispanics, and 2 Asians were included in the study group. A penile urethral swab was

collected for *N. gonorrhoeae* testing and for a Gram-stained urethral smear from each man who attended the STD clinic. A second swab was collected and placed into a *C. trachomatis* cell culture transport medium. The results of diagnostic *N. gonorrhoeae* tests and *C. trachomatis* culture were obtained from the clinic record. Samples from subjects who were HIV positive were not used. Both the University of Alabama at Birmingham Institutional Review Board and the Quality Improvement Office of the Jefferson County Department of Health approved this study.

Urethral specimen collection

Dacron-tipped swabs with stainless-steel shafts were used to obtain specimens of the anterior urethra and the penile *fossa navicularis*. The urethral swab was inserted into the urethral opening and withdrawn with a rotary motion. The swab specimen was placed into a glass vial (Wheaton Glass, Wheaton, Ill.) containing 1.5 ml of 2-SP transport medium (0.2M sucrose, 0.07M potassium phosphate monobasic, 0.1M potassium phosphate dibasic, fetal bovine serum [2%], and 12.5 units/ml nystatin [Sigma], 5 μg/ml gentamicin [Sigma], and 12.5 μg/ml vancomycin [Sigma], pH 7.6).

Primary urethral epithelial cell culture

Collected specimens were refrigerated for up to 24 h until a diagnostic cell culture was initiated. After the specimens were used for cell culture set-up, they remained refrigerated. Following diagnostic Ct cell culture inoculation, the remaining 2-SP transport medium with swab specimen was vortexed (VWR multitube Vortex, PLACE) for 2 min at a setting of seven. A 700-μl aliquot of the specimen was transferred to a conical 1-ml cen-

trifuge tube and was centrifuged for 10 min at 100 × *g*. The pellet was vortexed for 5 s along with the remaining residual liquid in the tube. An additional 300 µl of epithelial growth medium (Dulbecco modified Eagle's medi-um [DMEM] and Ham's F-12 [F12] 50/50 mix with HEPES and L-glutamine [Mediatech, Herndon, Va.], 5% heat-inactivated fetal calf serum [Mediatech], 26 µg/ml bovine pitui-tary extract [Clonetics, San Diego, Calif.], 5 µg/ml epidermal growth factor [Sigma, St. Louis, Mo.], 0.5 µg/ml hydrocort-isone [Sigma], 10 mM isoproterenol [Sigma], 5 µg/ml apo-transferrin [Sigma] and anti-microbials [see above]) was added to the cell suspension. Cells were first seeded in dup-licate (150 µl each well) onto a collagen Type IV-coated 96-well plate (Biocoat® Cell Environments™, Becton-Dickinson Labware, Bedford, Mass.). After the specimens were seeded onto 96-well plates, they were incubated at 37°C in a 5% CO_2 humidified incu-bator. Plates were visually inspected weekly. If cells were adherent and multiplying, the epithelial growth medium was changed as early as possible to enhance cellular prolif-eration. Cell cultures estimated to be confluent were treated with trypsin-EDTA, washed with plain DMEM/F12 medium, and sub-cultured. Following a period of time (approx-imately 1 month) when near confluence was detected in T-25 flasks, the cells were again treated with trypsin-EDTA and transferred to a T-75 flask. The cell-expansion process required from one to several months before flasks were confluent with cells.

ATCC urethral cell-line culture

A human female carcinoma-associated urethral cell line identified as Hs 769.T was obtained from ATCC (Rockville, Md.). The cell line was grown in the same medium as

the primary cells, using the same incubation conditions and evaluation parameters. This cell line has not previously been characterized or described in the literature.

Morphological examination and transmission electron microscopy

Cells were washed with PBS and fixed with 1% glutaraldehyde in PBS (pH 7.0). The cells were postfixed with 1% cacodylate-buffered osmium tetroxide for 1 h. Post section staining was performed with uranyl acetate and calcined lead combination stain (Hyatt). The ultra-thin sections were examined and photographed with a Philips CM-10 electron microscope (Einhoven, Holland) operated at 80 kV.

Immunohistochemical analysis

Cells were grown on single well slides (Nunc, Napierville, Ill.) and washed in PBS. The cells were fixed with 1% paraformaldehyde for 30 min at 37°C and washed with PBS. Slides were blocked with 50% goat serum for 30 min at 37°C. For cytokeratins, the slides were stained with a monoclonal anti-Pan cyto-keratin (cytokeratins 5, 6, and 8, [Sigma]). Some cells were stained with a monoclonal anti-human epithelial cell-specific antigen (Sigma). The negative control for these stains was a monoclonal anti-human fibroblast surface protein (Sigma) or no antibody at all. Following 1 h incubation at 37°C with the selected primary monoclonal, the slides were washed. Next, the slides were stained with a FITC-labeled, goat anti-mouse monoclonal (Chemicon International, Temecula, Calif.). The nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole-2HCl). Stained cells were viewed with an AX70 Olympus microscope equipped with a digital camera. Photo composites were managed using the Zeiss Optivert software (Zeiss).

Cytokeratin analysis by western blot

For non-subjective confirmation of cytokeratins associated with the epithelial cells, western blots were performed with monoclonal antibodies to cytokeratins. A human fibroblast cell culture was used as the negative control (16). After primary growth medium was removed, the cells were washed with PBS. The proteins were extracted with lysis buffer (0.5% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 10mM tris-HCl at pH 7.35) and placed in boiling water for 5 min. Specimens were diluted in tris-glycine-SDS buffer and run on a 12% Tris-glycine gel. The gel was transferred to an Immobilon-P membrane (Millipore, Bedford, Mass.). After transfer, the membrane was blocked with Superblock™ (Pierce, Rockford, Ill.) with 0.05% tween for 1 h at room temperature. Following a wash, the membrane was incubated with the mouse anti-human monoclonal anti-Pan cytokeratin 5, 8, and 9 (Sigma) in Tris-buffered saline for 2 h. The membrane was washed again and incubated with the goat anti-mouse IgG biotinylated secondary antibody. Next, the membrane was washed and incubated with Neutr-Avidin-HRP (Pierce) for 1 h. The bound antibodies were detected by the enhanced chemiluminescent (ECL) method with Super Signal (Pierce) and developed on Kodak BioMax film (Kodak, New York, N.Y.).

Growth kinetics

To determine the doubling time in the cell line and several primary cells, a 48-well plate (Costar, N.J.) was seeded with approximately 1×10^4 cells and harvested with Accutase® (Innovative Cell Technologies, La Jolla, Calif.) every 24 h for 1 week. The cells were stained with the vital stain trypan blue (Sigma) and counted in a hemocytometer.

C. trachomatis infection study

Primary urethral epithelial cells, as well as the urethral cell-line were grown in 48-well plates. In order to determine whether the cells could be infected with Ct, plates were first washed with PBS, and then the serovar E (Bour strain, ATCC) was added. The infecting chlamydial strain was diluted in 1 M sucrose phosphate glutamate (SPG) and added to a 96-well plate in duplicate wells for 2 h. The number of organisms per cell was set at a multiplicity of infection (MOI) of approximately 100. Control cells were mock infected in a similar manner but without the infecting strain added. The plates were rotated and rocked every 10-20 min for the 2-h period. Next, the cells were washed twice with plain DMEM/F12 medium. Growth medium (DMEM/F12 supplemented with 5% fetal calf serum) was added to the freshly washed cells. Following 72 h of incubation at 37°C in a humidified 5% CO_2 incubator, the medium was aspirated, and then the cells were fixed and permeablized with methanol. The cells were stained with a FITC-labeled monoclonal antibody against chlamydial LPS (Bio-Rad, Redmond, Wash.). The stained control cells and chlamydia-infected cells were examined with an inverted fluorescence microscope (Zeiss).

Comparison of patient primary positive *C. trachomatis*-culture in Hs769.T cells with McCoy mouse fibroblast cells

Results of 111 specimens from routine cell culture in McCoy mouse fibroblast cells that showed the presence of Ct in male urethral swabs and female cervical swabs (9) were compared to another culture method. The second culture method used discrete wells of Hs769.T urethral epithelium monolayers and discrete monolayers of McCoy mouse fibroblasts. These cell types were seeded into a 96-well plate in alternating rows and allowed to grow for 24 h. Unlike the routine culture McCoy cells that are pre-treated with DEAE-

Dextran, neither of the cell types used in the second method were pre-treated with anything prior to specimen inoculation. The medium was removed and one well of each cell type was inoculated with 100 µl of swab specimen contained in 2-SP transport medium. The 96-well plate was centrifuged at 100 × *g* at 37°C for 1 h. The samples were aspirated from the plate, and 200 µl of growth medium (DMEM/Ham's F12, 50/50 mix, epidermal growth factor 0.5pg/ml, and 10% fetal bovine serum) was added. The plate was incubated at 37°C with 5% CO_2 in air for 72 h. Following incubation, the cells were fixed with methanol and stained with a FITC-labeled anti-chlamydial LPS monclonal antibody (Bio-Rad). The inclusion-forming units from each of the positive wells (n = 12) with the two different cell lines were counted. The counts were compared to each other and to the inclusion-forming-unit count obtained from the routine culture system. Inoculation of specimens in the experimental method, staining, and reading of the plates occurred within 1 h from the time the first routine cell cultures were initiated.

Statistical analysis

Statistical analysis was performed using Mann-Whitney *U* test with InStat for Macintosh computers (Graphpad Software, Inc. San Diego, Calif.). Comparisons were considered significant at $P < 0.05$.

RESULTS

Primary urethral epithelial cell culture

From the 311 urethral specimens evaluated, 29 (9.3%) of all urethral swab specimens were capable of generating an ongoing primary cell culture. Attachment and growth of the primary urethral epithelial cells were noted only after a minimum period of 10 days for all successful primary cell cultures (Fig. 1). The average time for establishing cell

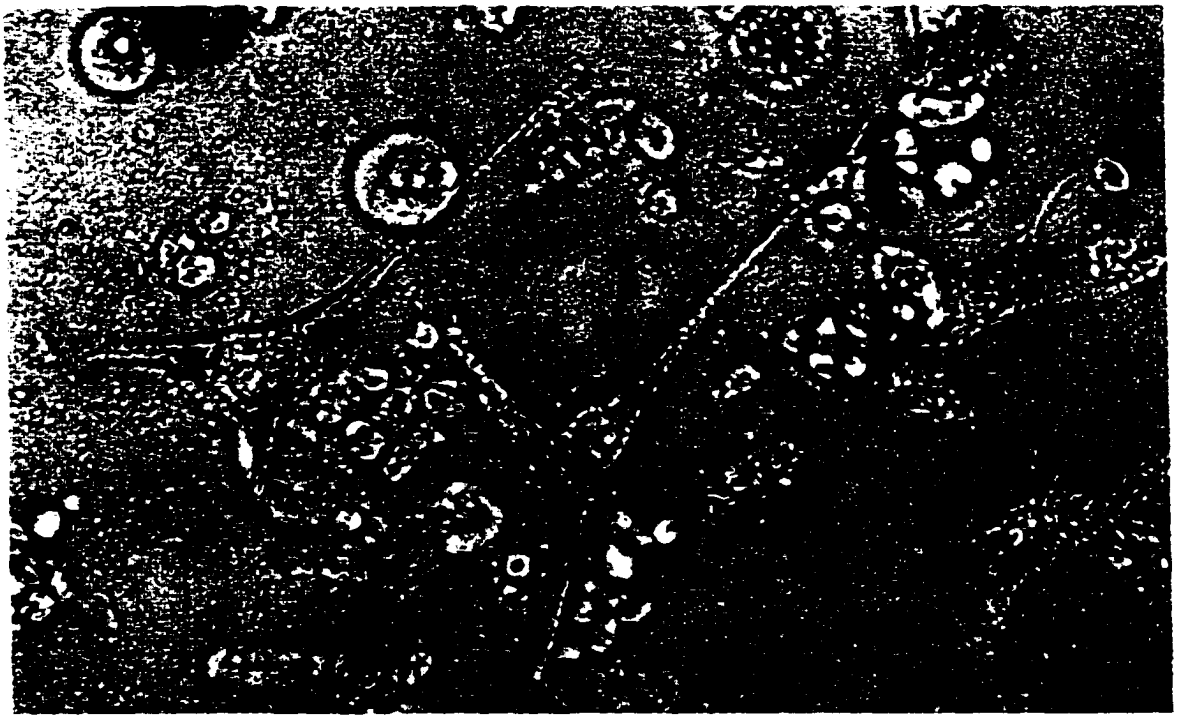

FIG. 1. Photomicrograph of primary male urethral epithelial cells. Initial attachment of primary male urethral epithelial cells are shown after 10 days in a well of a 96-well plate coated with a collagen IV matrix. Note the pseudopodial extensions that have extended from the cell body to make attachment to the substrate. Magnification ×400.

growth and proliferation of the primary cells was approximately 2 weeks from the time of plating.

ATCC urethral cell-line culture

Initial morphology of the cells in the ATCC transport medium showed cells with a diffuse, very long, spindly, filamentous, and fibroblast-like appearance. After an extended exposure (3 months) and multiple, primary cell-culture medium changes, the cellular morphology progressed through a transition of morphologies. Islands of normal-appearing epithelial cells that characterized this transitional state were surrounded by the undifferentiated, filamentous-type fibroblast cells (Fig. 2). The transitioned cellular morphology (Fig. 2) was similar in appearance to that observed in the primary urethral epithelial cells (Fig. 3A).

Morphological examination and TEM results

Cells from both the primary cell culture and the ATCC cell-line (Fig. 3A and 3B) showed normal epithelial-type morphology. A monolayer of cobblestone-pavement-appearing cells, punctuated by single spherical cells set between the margins of cellular joints, lined the bottom of tissue culture flasks.

Transmission electron microscopic evaluation revealed that the cells were most consistent with squamous epithelia in a complete monolayer (Fig. 4A). The cells most prevalent morphological feature was described as non-fibroblastic. The cells did not display tight junctional complexes. Internal structures were intact and well developed.

FIG. 2. Photomicrograph of Hs769.T cells in initial culture. Islands of well-defined female carcinoma-associated urethra epithelial cells surrounded by less-defined fibroblast cells are shown. These Hs769.T cells from ATCC contained a mixture of somatic fibroblasts and epithelial cells. Magnification ×200.

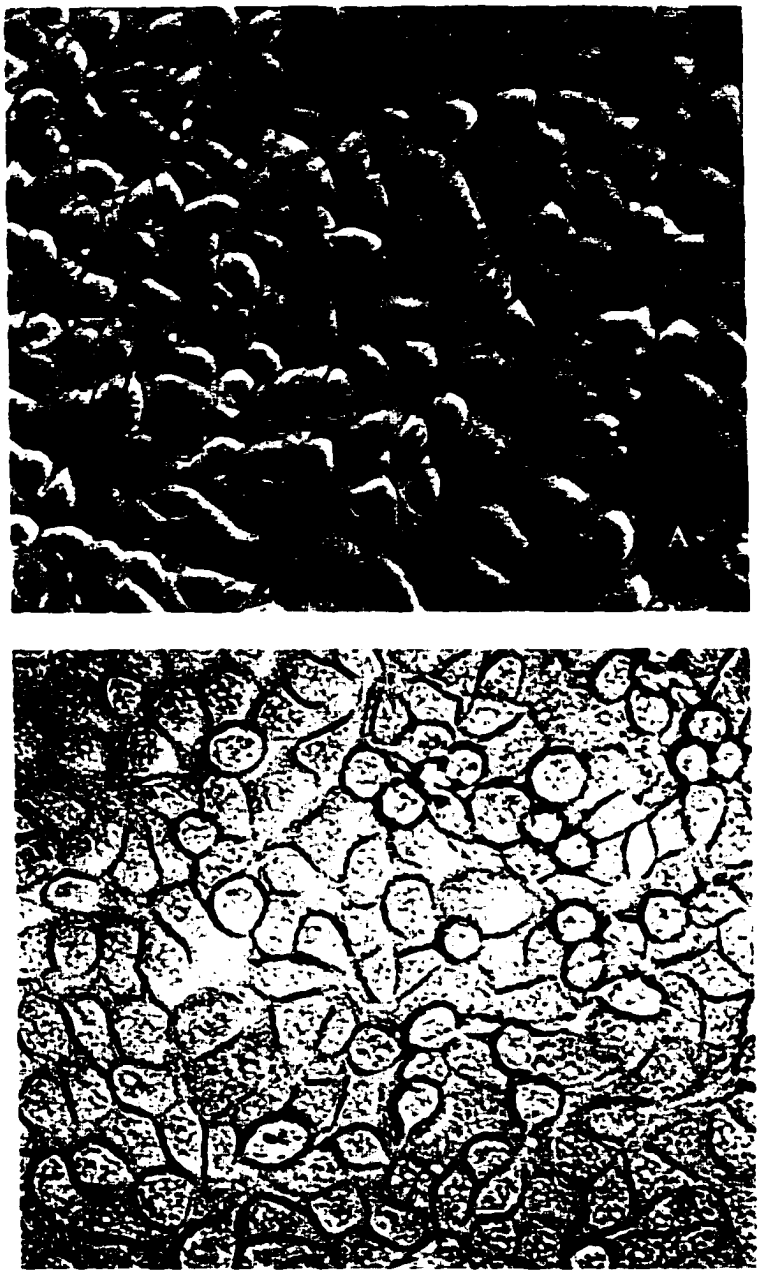

FIG. 3. Urethral epithelial cells. (A) A phase-contrast photomicrograph of primary male urethral epithelial cells. (B) A brightfield photomicrograph representing the morphology of the Hs769.T female carcinoma-associated urethral cell line. Both of the representative cultured cells from the primary specimens and the cell line show a cobblestone-like appearance that is equated with epithelial cells. Magnification ×400.

appearing cells, punctuated by single spherical cells set between the margins of cellular joints, lined the bottom of tissue culture flasks.

Transmission electron microscopic evaluation revealed that the cells were most consistent with squamous epithelia in a complete monolayer (Fig. 4A). The cells most prevalent morphological feature was described as non-fibroblastic. The cells did not display tight junctional complexes. Internal structures were intact and well developed. The nucleus was usually located in a polar position. Golgi was complete and well defined. Rough endoplasmic reticulum was modest and normal (Fig. 4B).

Immunohistochemical analyses

Both the ATCC cell line (Fig. 5A) and the primary urethral epithelial cells (Fig. 5C) stained positively for the Pan-cytokeratin antigens and also for cytokeratin 19. The control slides that were treated with an irrelevant antibody showed no staining (Fig. 5B and 5D). Cells taken from the primary culture flasks showed a mixture of cells that were both positive and negative for the fibroblast cell surface marker (Fig. 6).

Cytokeratin analysis by western blot

Results of the western blot provided proof that the cells were epithelial cells because they were positive for Pan cytokeratins 5, 6, and 8 (data not shown). Cytokeratin 19 was also present, which indicates that theses cells were either of a more immature state or were more basal in nature (Fig. 7).

FIG. 4. Transmission electron microscope (TEM) photomicrographs. (A) A TEM photomicrograph showing a monolayer of primary male urethral epithelial cells. Monolayer cultures of primary urethral epithelial cells did not produce tight junctions. Magnification ×1,200. (B) Transmission electron microscope photomicrograph of a primary urethral epithelial cell showing intact Golgi and an eccentric nucleus. Magnification ×3,700.

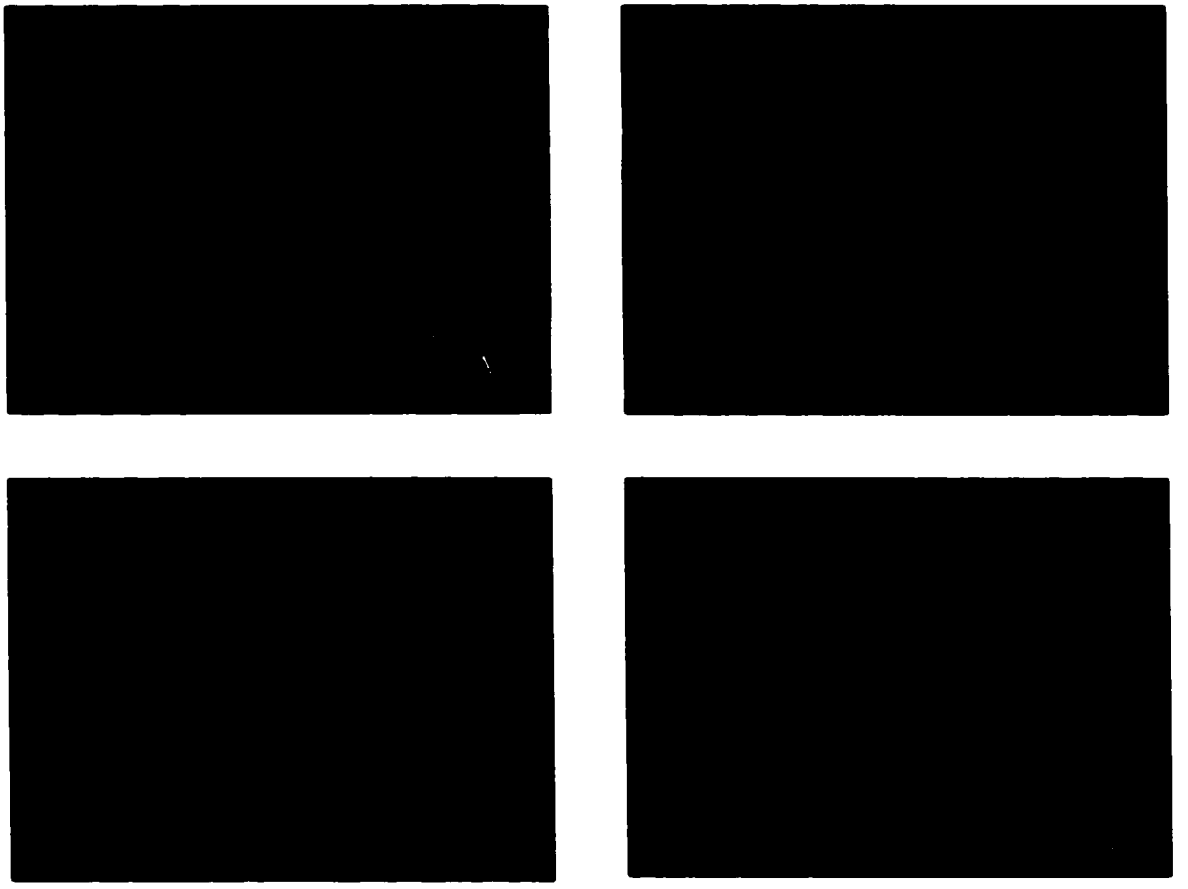

FIG. 5. FITC-labeled fluorescent stain of anti-pan-cytokeratin and anti-fibroblast surface antigen. (A) Hs769.T cells stained positively with the FITC-labeled (apple-green color) anti-pan-cytokeratin as well as did the (C) primary urethral epithelial cells. The pan-cytokeratin included antibodies to cytokeratins 5, 8, and 9. (B and D) The same cell types did not stain positively for the presence of fibroblast surface antigen thereby they acted as a negative control. DAPI was used to stain the nucleus blue.

Growth kinetics

The growth curve for the Hs769.T cell line demonstrated a doubling time that was consistent with an approximately 24-h time frame for up to 5 days and then a decrease in the number of viable cells after 5 days (Fig. 8). The growth kinetics for the primary cell cultures showed similar expansion times. Variable extremes were demonstrated from donors with a low maximum count at 5 days of 2×10^5 cells per well in a 48-well plate to the highest maximum cell count of approximately 4×10^5 cells per well at about 5 days (Fig. 9). The number of cell passes has been continuous, with some primary cells being passed up to 43 times as of this writing.

Infection of cells with *C. trachomatis*

Cells were infectable with Ct, serovar E (Fig. 10). An MOI of approximately 100 generated infections in more than 75% of all cells in a single well from a 96-well plate. All infected cells showed inclusion forming units with the similarities of Ct-infected fibroblast cells.

Cell type *C. trachomatis* culture comparison

Positive and negative results of routine Ct-culture method for urethral and cervical swab specimens from the 111 patients agreed with the comparison cell culture method results. The total number of 12 (10.8%) patients were positive for Ct. For those specimens that were Ct-positive, the number of IFUs counted in the Hs769.T cells were dramatically increased over the number of IFUs seen in the McCoy mouse fibroblast cells, regardless of the method used. The quality of the cultures was similar (cell morphology,

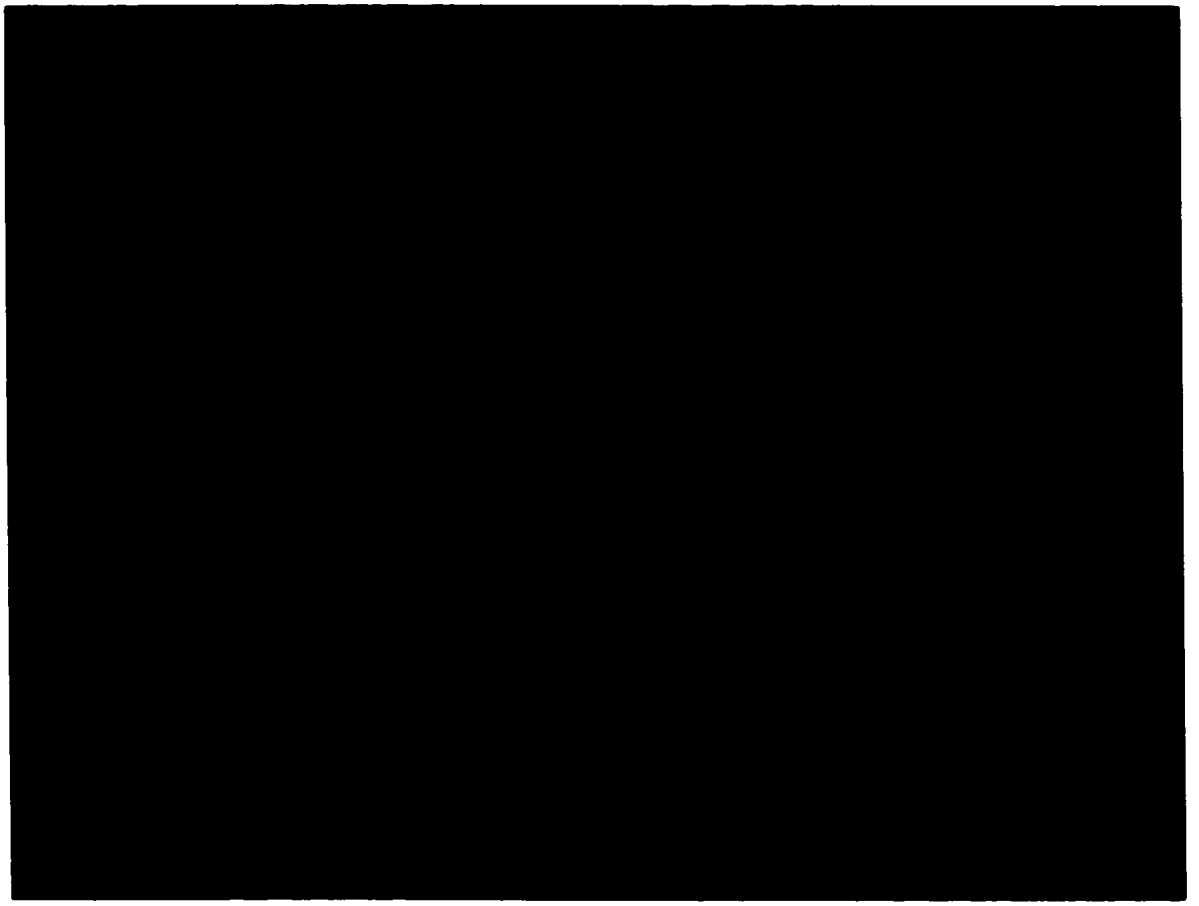

FIG. 6. Photomicrograph of original Hs769.T cells. This photomicrograph depicts a mixed cell population. The mixture was indicated by the presence or the absence of the FITC-labeled monoclonal antibody specific for fibroblast surface antigen (FSA) marker. An FSA-positively-stained cell is shown at the top of the frame indicating a fibroblast. The cell pictured at the bottom of the frame did not stain positively for FSA, indicative of other cells that were present in the mixture.

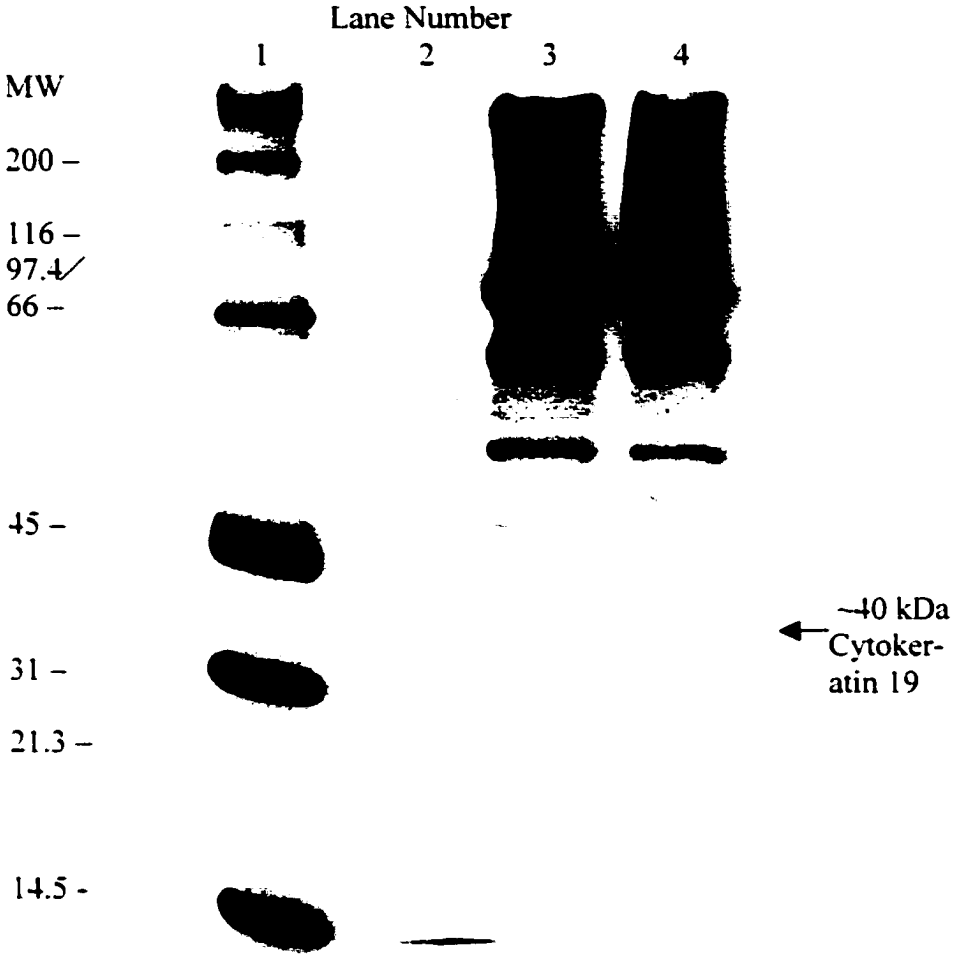

FIG. 7. Western blot analysis for cyokeratin-19. Results show the presence of cytokeratin-19 in the male primary human urethral epithelial cells (PHUEC) and the Hs769.T female carcinoma-associated urethra cell line. Lane 1 represents the protein standard, lane 2 represents the negative control (human fibroblast), lane 3 Hs769.T cell line, and lane 4 represents a PHUEC sample. The Hs769.T cells and the PHUEC cells were positive for cytokeratin 19 as indicated by the band at ~40 kDa molecular weight marker. Cytokeratin-19 is usually associated with a more immature cell, indicating that these cells may be more basal in origin.

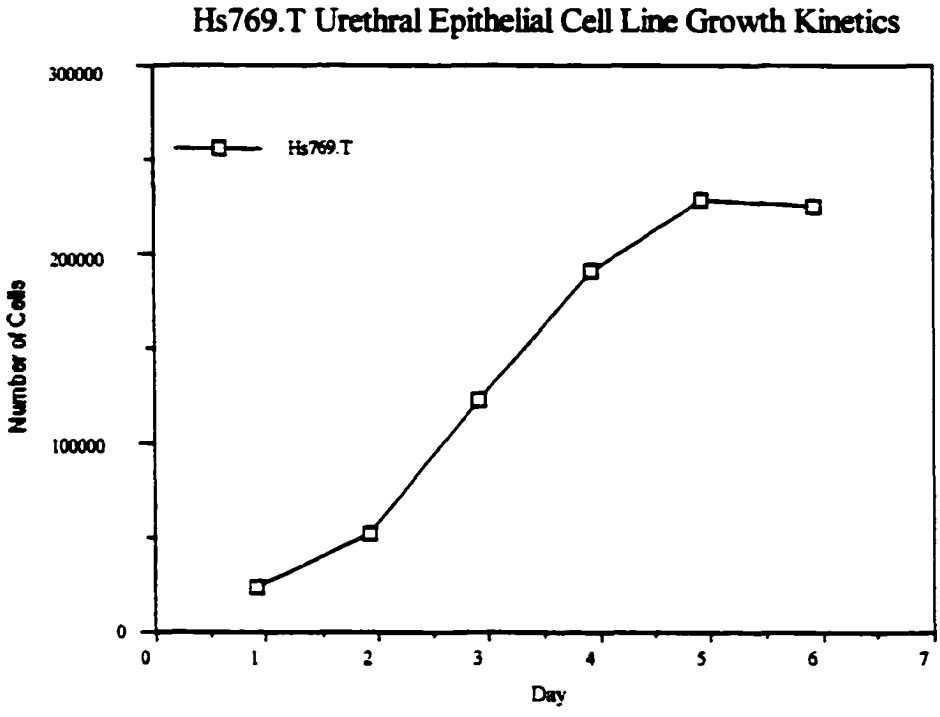

FIG. 8. Growth kinetics for Hs769.T. This female carcinoma urethral epithelial cell line was initially seeded at 10,000 cells per well in a 48-well plate. Following Accutase® treatment, the cells were stained with trypan blue exclusion dye and counted using a hemocytometer. Counts were performed daily for 1 week. Results show a doubling rate that approximates a 24-h time span for days 1 through 5 and afterwards a decrease in viability. The data represent one of three similar evaluations.

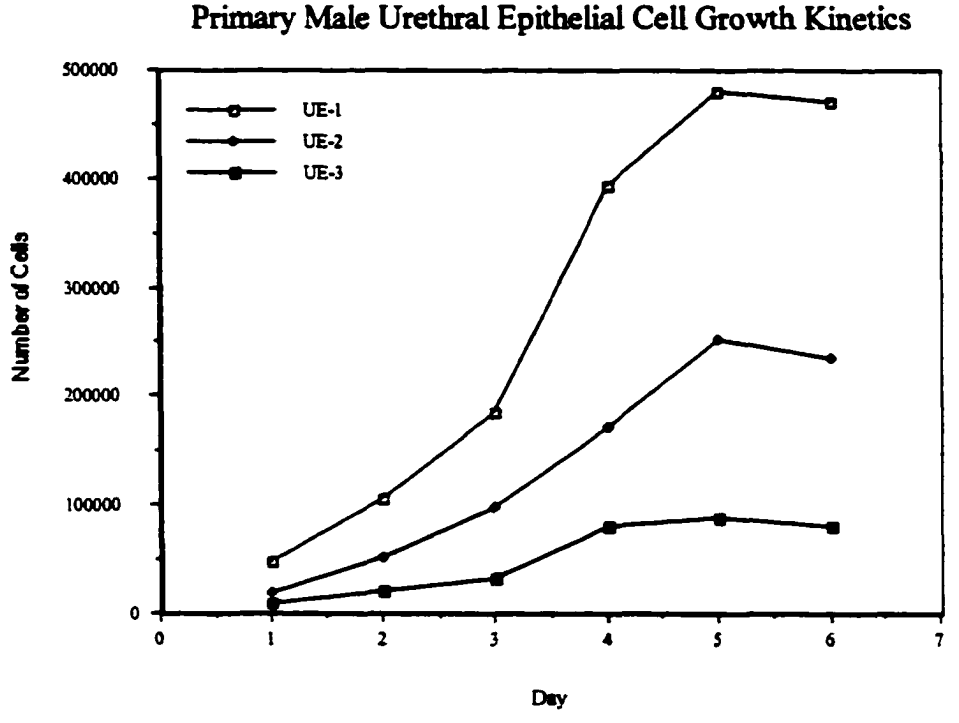

FIG. 9. Growth kinetics of primary male urethral epithelial cells. For daily counts, the cells were treated in a similar manner as the Hs769.T cell line. A variation in growth kinetics of primary cells (see UE-1 and UE-3) would imply that differences in cellular kinetics of these cells may exist. In evaluation of infected cells, these primary cells need to be evaluated as well as cell lines to establish overall cellular responses generated to infecting organisms.

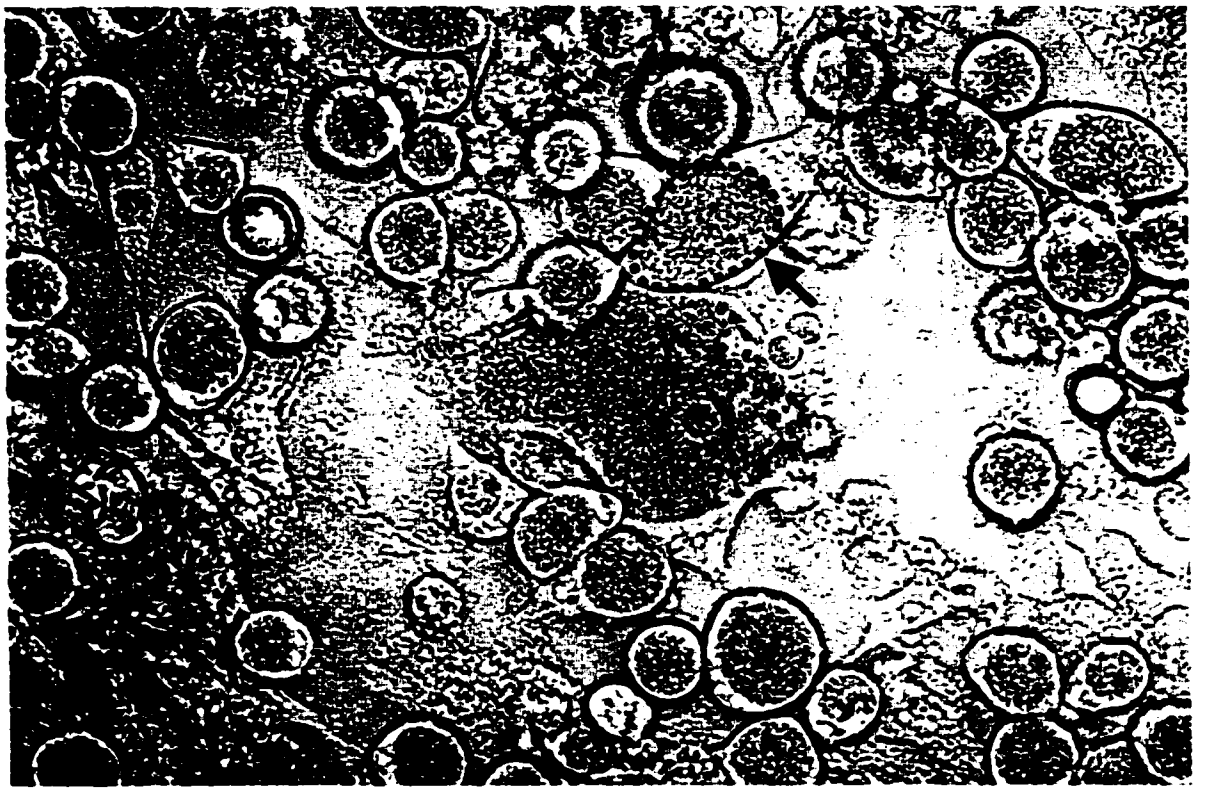

FIG. 10. Photomicrograph of Ct-infected cells. The photomicrograph shows 3-day-old primary male urethral epithelial cells infected with Ct. Cells were infectable with Ct-serovar E (Bour strain) at 100 MOI per well of a 96-well plate. More than 75% of all cells were infected. Note the outlined intracellular inclusion indicated by the arrow. Magnification ×400.

color, staining intensity) but the numbers of IFUs observed and counted were statistically different (Fig. 11) using the Mann-Whitney *U* test ($P < 0.0005$).

DISCUSSION

Epithelial cells of the male urogenital tract have previously been cultured from semen (10), but their origin was not defined. These semen-derived epithelial cells could only be maintained in culture for less than a 2-month period. Other than the semen-associated, primary epithelial cells that have been cultured *in vitro*, the only primary urethral epithelia culture method that has been described used urethral explants for seeding and establishing a limited cell-culture system (3). The present method utilized existing swab specimen samples to initiate and establish an ongoing primary urethral epithelial cell-culture system. This method should make primary culture of urethral epithelium available to more investigators for the evaluation of male urogenital/STD pathogenesis with an unlimited number of pathogens. The commercially available urethral cell line also makes for a suitable culture system that allows comparison of experimental results between the primary cell culture and the cell line. Because the culture of primary urethral epithelial cells from normal individuals will most likely have some differing experimental results (see primary cell growth curve, Fig. 9), the cell line will provide an established baseline for many comparison studies. Since these primary cell monolayers mimic the native epithelial morphology of the urethral tissue, this cell culture system lends itself to many investigative uses. The fact that Ck-19 was found as an epithelial marker in the male urogenital tract (13) may suggest that these cells are more basal in their origin (8). Cells with stem-cell

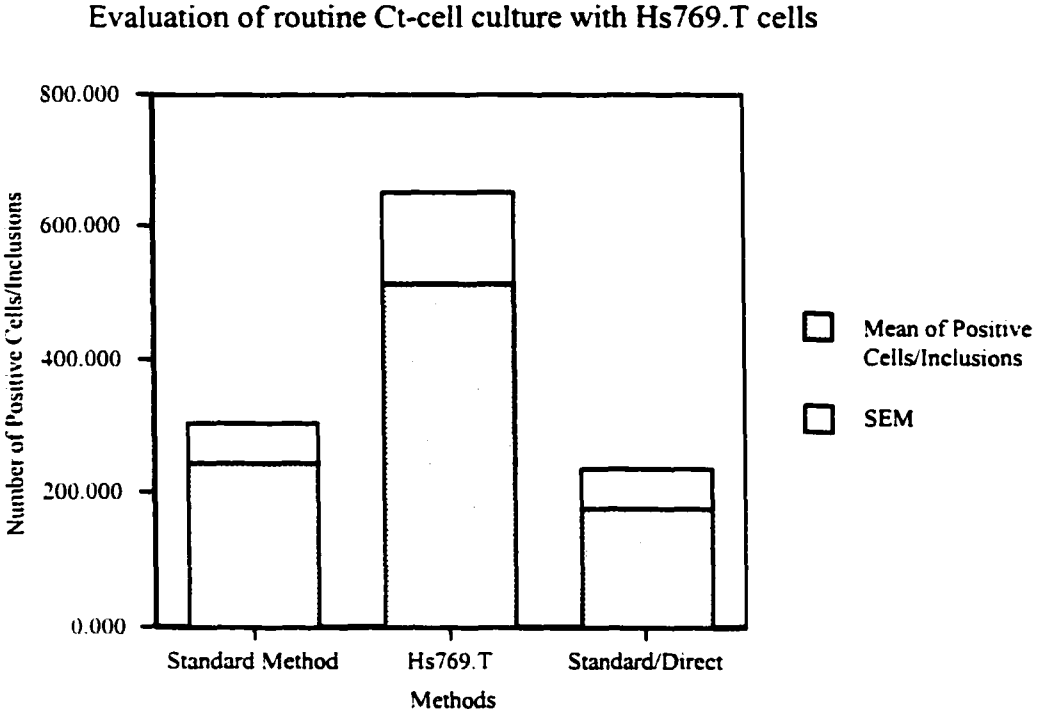

FIG. 11. A direct comparison between the standard *C. trachomatis* (Ct) cell culture for primary clinical specimens and the Hs769.T cell line. The total Ct inclusion counts in cell culture from Ct-infected patients were used to measure quality of culture outcome. Inclusion counts from three cell culture methods were evaluated. Samples of patient endocervical and penile urethral swabs (n = 111) were inoculated onto the monolayers of cells. After ~72 h the cells were stained with FITC-labeled monoclonal antibodies to *C. trachomatis* LPS. Direct comparisons were made between the standard method and the Hs769.T cells, and between the standard-direct method and the Hs769.T cells. The standard culture McCoy fibroblast cells were pre-treated with DEAE-Dextran, but the Hs769.T cells were not. In a similar fashion, additional McCoy fibroblasts cells were inoculated in the same manner as the Hs769.T cells (This was considered the direct method). There were 12 positive samples out of 111 that were positive for Ct. There was no significance detected between the standard method and the Hs769.T cell culture (P = 0.12). However, the direct comparison to the cells undergoing no pre-treatment showed a significant increase in the numbers of positive cells from infected patients (P = 0.03). These results may suggest that the Hs769.T cell are as responsive to clinical-sample infections as the pre-treated McCoy fibroblast cells.

-like qualities were most likely present in the populations of cells evaluated due to the number of times that these cells have been passaged (>43 times at this writing).

ACKNOWLEDGMENTS

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PRIMARY URETHRAL EPITHELIAL CELLS DO NOT PRODUCE CYTOKINES AFTER EXPOSURE TO *C. TRACHOMATIS*

by

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Format adapted for dissertation

ABSTRACT

The pathogenesis of *Chlamydia trachomatis* (Ct) in the male genitourinary tract has been little studied. We examined whether primary epithelial cells from the male urethra and other epithelial cells from other sites in the male urogenital tract produced cytokines following exposure to Ct. Cell supernatants of Ct-infected and -uninfected cells were collected at time zero, 6 h, and daily for 5 days. Cytokine levels of interleukin (IL) -1α, IL-1β, IL-6, IL-8 and transforming growth factor-beta (TGF-β) were measured by enzyme-linked immunosorbant assay (ELISA). The primary urethral epithelial cells and cells from a urethral cell-line constitutively produced only TGF-β, but no other cytokines. The prostate PC-3 cell line produced detectable levels of all cytokines with increases in IL-1α, IL-1β, and IL-6 when infected with Ct. The kidney 293 cell line produced only elevated levels of IL-8 when infected with Ct.

INTRODUCTION

Chlamydial trachomatis (Ct) infections of the genital tract comprise the largest group of bacterial sexually transmitted diseases (STDs) in the United States. These type Ct infections together with the Ct-associated trachoma are a problem across the world. Understanding the human immune response to these infections will lead to better understanding of the disease process and possibly help to generate new targets for potential vaccine candidates.

For these infections, as well as numerous others, the targeted entry for pathogens is the mucosal epithelium. Epithelial cells are the first line of defense that aid in preventing the entry of microorganisms across the external barrier. These cells also initiate the ex-

pression of innate immune defenses such as cytokines. Cytokines are released to recruit additional immune help when threatened with active invasion by bacterial pathogens (10). Surveys of cytokine production by female-associated cell lines (HeLa, SiHa), colon cell lines, and primary endocervical cells infected with different serovars of Ct (D, I, and L2) have reported increases in the cytokines IL-1α, granulocytes-monocyte colony-stimulating factor (GMCSF), growth-related protein alpha (GRO-α), IL-6, and IL-8 (18). In light of the published results, expectations might presume that all Ct-infected epithelial cells behave in a similar fashion. The results of the present experiments reveal that male and female urethral cells do not actively secrete cytokines in response to Ct, *E. coli*, or *N. gonorrhoeae* infections. Not only do these cells not respond to bacteria but they also do not respond to exogenously added cytokines (IL-1α, IL-1β, IL-4, IL-10, and INF-γ). This type of epithelium is present in the human urethra and may represent additional primary cells for Ct infection in the human host, especially males.

MATERIALS AND METHODS

Cell lines and primary penile urethral epithelial cells

A female urethral carcinoma epithelial cell line was purchased from the American Type Culture Collection (ATCC, Rockville, Md.). This cell line was designated as Hs769.T. A human embryonal kidney epithelial cell line (293), as well as a human prostate epithelial cell line (PC-3) were also obtained from ATCC and used in the Ct-infection assays. Primary penile urethral epithelial cells were obtained from swab specimens submitted for Ct culture in transport medium from 6 men. These cells were grown on collagen-IV-treated 96-well plates, expanded in T-25 flasks until near confluence, and trans-

ferred to T-75 flasks. The cultured cells were cultured through several passages until the quantity of cells was sufficient to include in the following described experiments. All cells were grown in maintenance medium (DMEM/Ham's F12 [50/50 mix, Cellgro, Va.], 10% fetal bovine serum [FBS], 5 ng/ml epidermal growth factor, and antibiotics [mycostatin 12.5 units/ml, gentamicin 5 μg/ml, and vancomycin 12.5 μg/ml, [MGV]) incubated at 37°C with 5% CO_2 in air. Cells were detached with trypsin-EDTA, and the cells were examined on a hemocytometer using the trypan-blue dye exclusion to determine percent cell viability. Epithelial cell viability and cell counts were visually evaluated on a Leitz Laborlux microscope.

The Quality Improvement Office of the Jefferson County Department of Health and the University of Alabama at Birmingham's Institutional Review Board approved the current study.

Ct serovar E

A Ct Serovar E (Bour strain) was purchased from ATCC and grown in DEAE-Dextran-treated McCoy mouse fibroblasts in a T-75 tissue-culture flask with chlamydia growth medium (RPMI 1640, 10% FBS, 0.18% glucose, HEPES, NaOH, 1 μg/ml cycloheximide [Sigma, St. Lois, Mo.] 12.5 μg/ml mycostatin [Sigma], 5 μg/ml gentamicin [Sigma], 12.5 μg/ml vancomycin [Sigma]). The Ct serovar E was grown in a 5% CO_2 incubator at 37°C for 72 h and harvested by scraping. The cells were lysed by sonication and centrifuged at 500 rpm for 10 min to remove cellular debris. Renograffin density purification of the elementary bodies (EBs) was performed by ultracentrifugation at 18000 × *g* for 1 h. Purified EBs were pelleted and placed in isotonic sucrose phosphate glutamate

(SPG), and then frozen at -80°C. Ct infectious titer was determined by serial titration onto the Hs769.T cell line. The cells were stained with a FITC-labeled, anti-chlamydial-LPS monoclonal antibody stain (Pathfinder™, Bio-Rad, Redmond, Wash.).

Ct serovar L2

The serovar L2 was purchased from ATCC and was directly inoculated onto the cells for infection studies. Determination of infectious titer was initiated at the same time as infection studies to determine the multiplicity of infection (MOI) or number of viable infectious units per host cell.

Infection protocol

Approximately 2×10^5 urethral epithelial cells were seeded into each well of a 48-well plate and incubated in 5% CO_2 in air at 37° C for 24 h. Epithelial cell monolayers were washed twice with Hank's balanced salt solution (HBSS) before addition of the serovar inoculum. The cells were infected with a MOI ranging from 0.02 to 200 in SPG in a volume of 200 µl per well. Plates were rocked every 15 min for 2 h at room temperature. The inoculum was removed and the monolayer was washed twice with HBSS. Each well was filled with 500 µl of growth medium (DMEM/Ham's F12 [50/50 mix, Cellgro, Va.], 10% fetal bovine serum [FBS]) and incubated. Infected cells in 48-well plates were incubated as before for up to 5 days.

Infection of Hs769.T cell line and primary cells with *Neisseria gonorrhoeae* and *Escherichia coli*

For cellular cytokine response analysis, the Hs769.T and the primary cell lines were infected with a high titer of *N. gonorrhoeae* (approximately 500 MOI). The same cell types were infected with *E. coli* at approximately 300 MOI for 1 h prior to adding antibiotic-(MGV)-supplemented medium containing gentamicin.

Addition of exogenous cytokines to tissue culture medium

Attempts to stimulate the Hs769.T cells and the primary urethral epithelial cells were performed by adding exogenous concentrations of IL-1α, IL-1β, IL-4, IL-10 and IFN-γ (R&D Systems, Minneapolis, Minn.) to the growth medium of the cell-culture wells.

Supernatant sampling and cytokine assays

Supernatants from wells of Ct-infected and -uninfected control cells were removed at time zero, 6 h post infection and each 24 h thereafter for 5 days. Supernatants were stored at -80° C until assayed. Cytokine ELISA assays were performed on the harvested cell supernatants to measure concentrations of IL-1α, IL-1β, IL-6, IL8, and TGF-β (Duo-sets, R & D Systems). The assays were performed according to manufacturer's directions.

Statistical analysis

Mann-Whitney *U* tests for statistical analysis were performed using Instat for Macintosh computers (Graphpad Software, Inc. San Diego, Calif.). The level of significance was placed at $P < 0.05$. All evaluations were considered as significant if the numerical values obtained from the appropriate statistical tests were equal to or below 0.05.

RESULTS

Ct-infected and -uninfected primary human urethral epithelial cells (PHUEC) and Hs769.T Cell Line

PHUEC from males and cells of the Hs769.T female urethral cell line did not produce any of the cytokines evaluated from supernatants during these experiments, whether infected with Ct at varying concentrations of inoculum from 0.02 MOI to 200 MOI or not infected. These cells did not produce detectable levels of IL-6 or IL-8 in response to infection with *E. coli* and *N. gonorrhoeae*. Cells were exposed to biological levels of IL-1α, IL-1β, IL-4, IL-10, and IFN-γ without any production of measurable cytokines. However, both the PHUEC and Hs769.T cells did produce constitutive levels of TGF-β (Fig. 1).

Ct-infected and -uninfected kidney cell line 293

The embryonal kidney cell line 293 was only minimally more responsive in cytokine production that the PHUEC and Hs769.T cells. When infected with Ct, the 293 cells increased their levels of IL-8 in the supernatant over the whole time course evaluated (Fig.

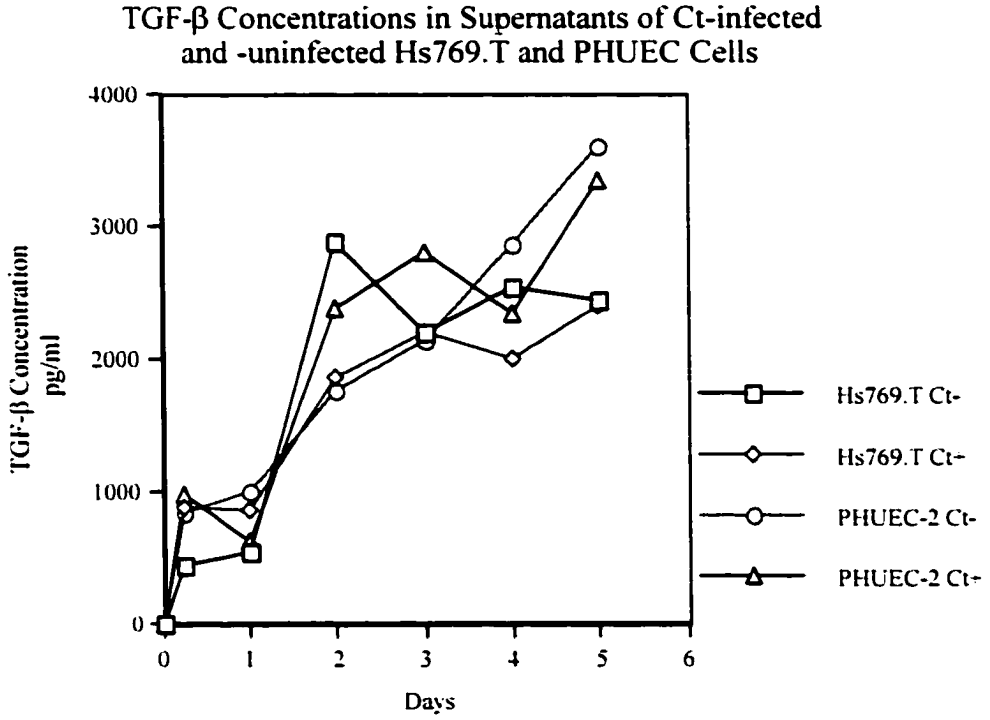

FIG. 1. Urethral cell TGF-β concentrations. TGF-β was found in both the cell line. Hs769.T and in the primary human urethral epithelial cells (PHUEC). Both showed similar levels with or without the presence of 2 MOI of Ct. TGF-β was constitutively expressed by all cells. and cumulative amounts showed a relatively moderate increase over time.

2). These uninfected cells had cumulative levels that increased during the measured time frame. The Ct-infected cells had from four to eight times more IL-8 in supernatants than that of uninfected cells.

Ct-infected and -uninfected prostate cell line PC-3

The most responsive cells to Ct-infection were the PC-3 prostate cells. These cells had detectable levels of IL-1α, IL-1β, IL-6, and IL-8 in their supernatants. Levels of IL-1α were increased for the cells infected with Ct from days 1 through 4 with a decrease on day 5. Nonetheless, the uninfected cells had no detectable levels associated with them during the whole time course (Fig. 3). Concentrations of IL-1β, increased from days 1 through 4 also with reduced levels on the fifth day (Fig. 4). Totals of IL-6 were above the 1200 pg/ml level for all cells regardless of infection status (data not shown). Concentrations of IL-8 were near a constant level for the Ct-infected as well as for the uninfected PC-3 (Fig. 5).

DISCUSSION

Persistent Ct infections in women have been a subject of much research and attention for several years. Numerous hypotheses have been formulated to explain the chronic Ct infections seen in women who develop pelvic inflammatory disease (PID) and in people with persistent ocular infections. These hypotheses and their supportive works include the active suppression of apoptotic stimuli in Ct-infected cell culture (8) and Ct-infected

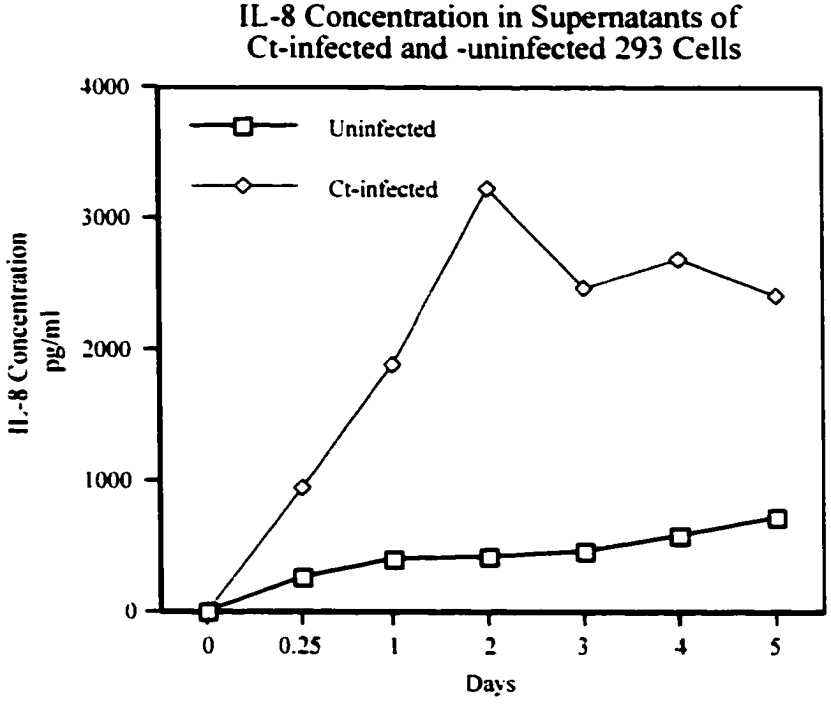

FIG. 2. The kidney cell line 293 and levels of IL-8. The 293 cells produced IL-8 when they were not infected. Constitutively produced IL-8 levels were low compared to the increased levels when the cells were infected with 2 MOI of Ct. The Ct-infected cells showed a steady rise from time zero to day 2 with the largest increase over background levels on day 2, as illustrated by the upper red line. On days 3 through 5, levels of IL-8 decreased but were approximately 5 times higher than levels produced by the uninfected cells.

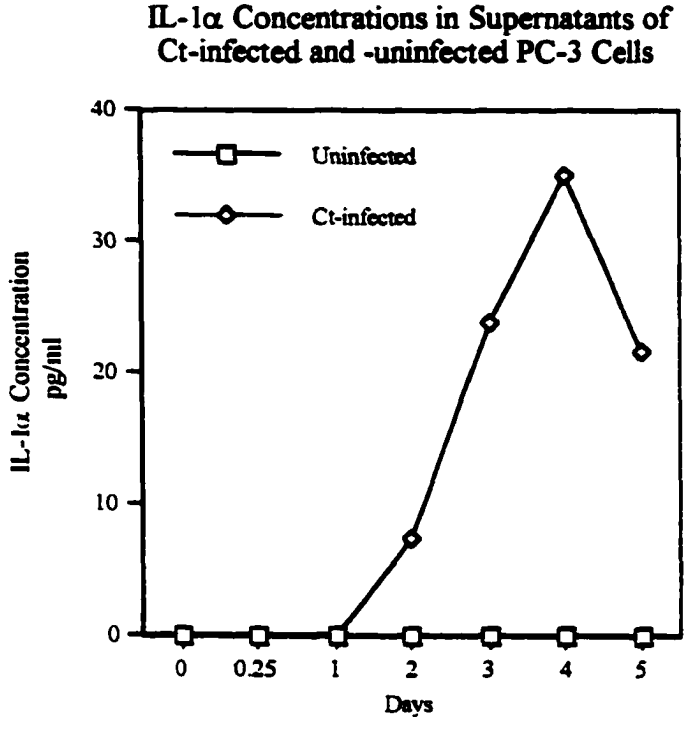

FIG. 3. IL-1α levels in PC-3 cells. The cytokine IL-1α maintained levels below detection limits for the prostate cell line PC-3 when not infected with Ct. However, dramatic increases were seen in the same cell line when infected with Ct. Beginning on day 2 and through day 4, levels increased. The IL-1α level for day 5 dropped, but may have been due to cell destruction.

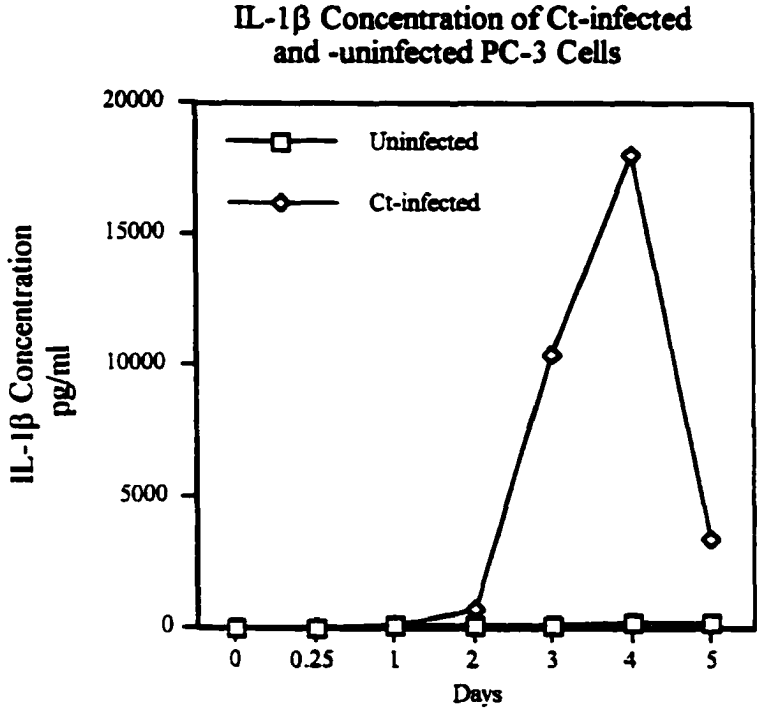

FIG. 4. IL-1β levels in PC-3 cells. For the PC-3 prostate cells, the concentration of IL-1β was increased on days 2 through 4 when infected with Ct. A dramatic decrease was seen on day 5 with only nominal levels observed when cells were uninfected with Ct. This cytokine might initiate the inflammatory process from the Ct-infected cells.

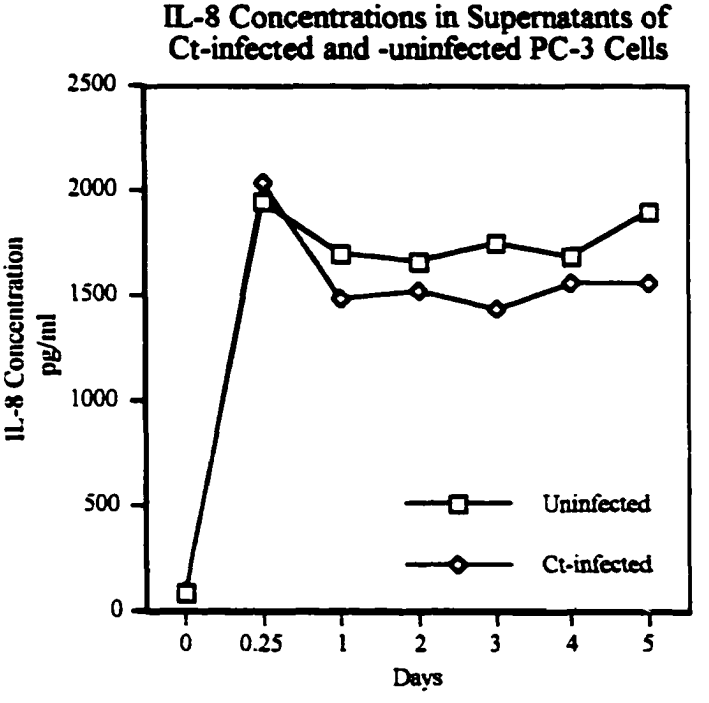

FIG. 5. IL-8 levels in PC-3 cells. Constitutive levels of IL-8 were expressed over the time course when the PC-3 prostate cells were and were not infected with Ct. No difference in the amounts of IL-8 between the two groups suggests that prostate cells are not a source for increased levels of IL-8 seen in the local evaluated fluids from the male urethra when infected with Ct.

monocyte-derived macrophages (13). In some serovars of Ct, the truncated tryptophan synthase gene may depress or inactivate the effects of IFN-γ and has been proposed as a method for persistent infections (20). Residual chlamydial-envelope antigens, which exists for weeks following antimicrobial treatment, have been implicated as a means for maintaining a long term and persistent infection (21). The total load of infectious organisms and duration of infection (4) has been attributed to persistence.

In vitro evaluations of persistent Ct infections have provided much information that has yet to be formulated into a comprehensive, realistic, and explainable fashion that can transfer the findings from these limited modeling systems to one that mimics the *invivo* pathology. Many Ct-infected cell-culture systems have been initiated to look at the mechanisms that may help in the evolution of persistence. Numerous experimental systems have been successful at giving logical and explainable results that can be interpreted as causative means for long-term Ct infections (1-3, 5-7, 12, 14-17, 19). Many studies have examined the proactive or reactive results that are manifested by Ct-infected cells, such as secreted cytokines or innate immune components. But few, if any studies, have looked at the inactivity of the host's cells following Ct infection. This study presents data that suggests that some primary cells of the male penile urethra and a commercially avail-able cell line from a carcinoma-associated female urethra may be infectable with Ct but unresponsive as part of active immune mediators of apparent infections.

The Hs769.T cells have never been evaluated in experimental research, yet they represent new and exciting findings with each experiment even when negative results are revealed. In the current study, except for TGF-β, cytokines were absent in the cell line's supernatants regardless of the Ct-infection status. TGF-β was constitutively expressed

during all evaluation times. The TGF-β levels were similar in both the uninfected cells and the Ct-infected cells for all MOI (range 0.02 to 200 MOI). Similarly, the male PHUEC cells reacted in a like manner, which corroborates the results obtained on the non-responsive cell line.

Most epithelial cells are understood to produce increased amounts of pro-inflammatory cytokines when exposed to antigenic stimuli such as LPS (9) and to cytokines, such as IL-4 (11). Nevertheless, these cells that are derived from the urethra appear to be very non-responsive to stimuli whether they be real bacteria (*E. coli* and *N. gonorrhoeae*) or other cytokines (IL-1α, IL-1β, IL-4, IL-10 and IFN-γ), or whether the cells are infected with an intracellular bacterium Ct. These cells may indicate a potential reservoir for hiding intracellular organisms from the host's immune surveillance. Intracellular organisms such as Ct have possibly exploited these epithelial cells due to their lack of response to establish initial infections.

As positive controls, the kidney 293 cells and prostate PC-3 cells exhibited greater levels of response, but not uniformly with all stimuli. Because this study investigated representative tissue further up the male urethra, these findings provide a more comprehensive evaluation of the male genital tract's response to Ct infection. These results represent the first time that either of these two specific cell lines have been shown to be infectable with Ct.

The results of these Ct-infection studies revealed a definitive difference in responses by primary urethral epithelial cells from males compared to primary endocervical cells from females, as described by Rasmussen et al. (18). While most of the serovars of the infecting strains from the previous study with female-associated cells were different from

the serovar E used in the present study, one would expect to see similar results from Ct-infected epithelia. With the prior female cell culture studies, the Ct serovar L2 had been included as the infecting agent with increased amounts of detectable cytokines. As a control in the primary urethral epithelial cell experiments, the serovar L2 was also used. However, even with the L2-serovar-infected primary urethral cells and the cell line, no detectable cytokines were measured. Differences exist in the genitourinary-reproductive tract anatomy of both sexes. Perhaps a real difference between the sexes also exists in the manner by which Ct infections are managed by the hosts.

In a previous study (see first publication), elevated levels of IL-8 were detected in the urethra of males infected with Ct. However, the cells evaluated in this series of experiments did not produce this cytokine. One can speculate that the source of IL-8 may be neutrophils or perhaps other cells not yet tested. While the source of IL-8 in the Ct-infected male urethra is still elusive, these results provide information that eliminates the primary urethral epithelial cells as the source of the increased amounts of IL-8.

In the present study, a potential reservoir was revealed that would allow Ct to hide from the host's immune system and continue to increase in number with only minimal detection. Apparently, the host's immune system responds to the presence of the pathogen's EBs only after the Ct has gone through a replicative cycle. With the potential for may cells to undergo asynchronous infections, many EBs could possibly be released at different times, and the number could grow to such an overwhelming infection that the immune response would be minimal when compared to the numbers and rapid uptake of these EBs by the host cells that the growth could promote further infection that goes undetected. The results of these studies may suggest that the urethral epithelial cells are a

primary target for initiating and maintaining a persistent infection that is invisible to the host's immune surveillance.

ACKNOWLEDGMENTS

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DISCUSSION

The development of a vaccine to prevent Ct-infection or lessen its severity is crucial. Progress towards the realization of a candidate vaccine can be accelerated if we understand the human immune response generated against this organism in the reproductive tract of males and females. Immunization in the vagina with antigens such as *Candida albicans* (90) or inactivated polio virus (64) has been shown to produce very modest local antibody responses. Additionally, immunization of the vagina with a more robust immunogen such as cholera toxin B subunit (CTB) elicited a similar antibody response, yet no immune responses were detected in mucosal-associated fluids located at distant locales from the primary site of immunization. Moreover, the local immune responses in the male genital tract of STD-infected men has not yet been reported.

Immunization of humans by the oral route generates low antibody responses in the genital tract. However, the oral administration of a vaccine combined with local, genital tract immunization, results in an increased specific antibody response in the genital tract. Based on studies conducted in experimental animals, rectal and intranasal immunizations proved to be efficient routes to induce immune responses in the genital tract. These alternative immunization routes for humans have caught the attention of many in the field of vaccinology. In females who have undergone rectal immunization with inactivated influenza virus, elevated levels of virus-specific IgA and IgG have been measured in cervical secretions at one month, with detection of both IgA and IgG measured at 6 months after immunization (22). It was reported that females who had been

rectally immunized with an attenuated strain of *Salmonella* had immune responses measured in vaginal washes that were less than those measured in females immunized with the same oral vaccine (39, 63). In the orally vaccinated females, the numbers of $\alpha 4\beta 7$-specific antibody-secreting cells in circulation were high (39), indicating a preferential migration to the gut. Using a combination of the two immunization routes, oral and then rectal immunization was shown to induce a more effective immune response in the genital tract (45). Vaccination with *Vibrio cholera* (killed vibrios and CTB) by the intranasal route was able to generate antigen-specific antibodies in the urogenital tracts of both males and females (6, 76). Influenza vaccine administered in humans by the intranasal route, was better than the oral one at generating antibodies in the genital tract (61). Intranasal immunization might be the best route for generating genital tract immune responses against STDs such as Ct because it induces both systemic and mucosal responses and because it is known that the genital tract Ig are derived from both the systemic immune system as well as the local mucosae.

Results from studies presented in this dissertation demonstrate that in the human male urethra, a less than vigorous immune response is mounted against Ct. From a survey of the Ct-infected men, the collected specimens were assayed for cytokines, immunoglobulins, and Ct-specific antibodies, and the following were found: (i) in Ct-infected men, of the eleven cytokines evaluated, the highest levels were found for IL-8 only; (ii) IgA, IgG, and IgM were higher in Ct-infected men than in the uninfected control group; and (iii) Ct-specific IgA and IgG antibodies were significantly higher in Ct-infected men than in the control group of men.

Also, the results of the first study demonstrated increased levels in S-IgA, IgA1, and IgA2 subclasses in Ct-infected men; these subclasses might play an important part in delaying or decreasing Ct-pathogenesis. Results from these studies will be important to further demonstrate cross talk or cellular interactions specific for cells of Ct-infected individuals and absent in uninfected cells.

The second study presented in this dissertation revealed that primary urethral epithelial cells can be grown from urethral specimen sampling swabs. Moreover, isolation and growth of these primary cells, as well as those of the commercially available female carcinoma-associated urethral cell line, provided this researcher's group and others with additional tools to evaluate the pathogenesis of Ct and other epithelial cell-associated STD agents. When the Ct-infected cell line or the primary cells are examined in light of innate immune responses that are contributed by these cells, a better understanding of the immune response develops, which might help in vaccine development.

The third study has helped to reveal that the epithelial cells from the urethra of the human male and a similar female urethral cell line are not responsible for secreting increased levels of the cytokines IL-1α, IL-1β, IL-6, IL-8, and TGF-β when infected with Ct. These cells are, by most standards, immunologically non-responsive to the infecting organism Ct. The results from all of these studies only strengthen the argument that the reproductive tract is designed primarily for reproduction, with limited availability to generate mucosa-associated immune responses against STD pathogens.

This dissertation has helped to shed more light on the immune responses generated against Ct in the male genital tract. However, more information derived from in-depth studies that evaluate the STD-infected genital tract of both males and females is necessary

to better understand whether vaccination might play a role in STD prevention and possible therapeutics. Although animal experimentation is necessary for evaluating the basic science of STD pathogenesis and immune responses, future research will need to target the involvement of humans based solely on the differences observed between the physiology of reproduction and the immune system in humans and animals. Inducing an immune response in the genital tract of both males and females against STD-related pathogens is necessary so that prevention and therapy to STD-related illnesses and their sequelae might be generated or improved.

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APPENDIX:

SUPPORTING DOCUMENTS

IRB APPROVAL FOR HUMAN SAMPLE USE IN RESEARCH

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☐ ORIGINAL ☐ FOLLOWUP ☐ EXEMPTION	☐ GRANT ☐ CONTRACT ☐ FELLOWSHIP ☐ COOPERATIVE AGREEMENT ☐ OTHER:_____	

4. Title of Application or Activity	5. Name of Principal Investigator,Program Director, Fellow, or Other
Mucosal Immunology of the Human Penile Urethra as Exposed to Sexually Transmitted Diseases	PATE, MITCHELL S

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☐ This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the common rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

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13. Name of Official: Marilyn Doss, M.A.	14. Title: Vice Chair, IRB
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RESEARCH SYNOPSIS

JEFFERSON COUNTY DEPARTMENT OF HEALTH

Title and Researcher	Summary of Activities	Centers Involved	Start Date	Projected Stop Date
Mucosal Immunology of the Human Penile Urethra as Exposed to Sexually Transmitted Diseases **Mitchell S. Pate, MT (ASCP)** Research Associate UAB Infectious Diseases ZRB 242 Birmingham, AL 35294 Phone: 934-4204 Fax: 975-7764	The purpose of this research is to identify, measure, and compare the biochemicals present in urethral swab fluid specimens and serum (due to be discarded) from males infected with *Chlamydia trachomatis* (CT) and uninfected (controls) with CT. The researcher will obtain a small sample (700 uL) from the stored culture samples collected at the JCDH. From this we will try to grow viable cells. If successful, we will infect the laboratory-grown cells with bacteria/viruses and compare the biochemicals produced within the laboratory-infected cells to natural infections. Overall, the objective is to identify a potential biochemical that may be useful in vaccine development against CT and possibly other sexually transmitted diseases. The researcher will maintain all information as confidential and will submit a final report to the Disease Control Director and the QI Director upon study completion.	Central Health Center-STD Clinic Recommend Approval R. Cudley Recommend approval 6/15/01 approved 6/21/01	July 2001	July 2006

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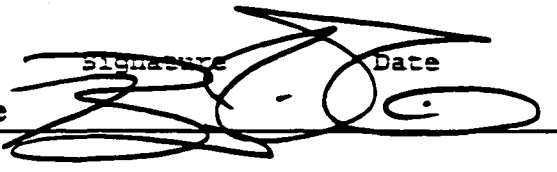
Brian Van Tine		11/18/01

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