INFECTION AND IMMUNITY, May 1995, p. 1754–1761 Vol. 63, No. 5
0019-9567/95/$04.00+0

Use of Tissue Culture and Animal Models To Identify Virulence-Associated Traits of *Haemophilus ducreyi*

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Received 1 December 1994/Returned for modification 18 January 1995/Accepted 7 February 1995

To identify virulence-associated properties of *Haemophilus ducreyi*, 34 strains of this sexually transmitted pathogen were evaluated for in vitro phenotypic characteristics of potential relevance to chancroid pathogenesis and for their ability to produce lesions in the temperature-dependent animal model for chancroid. Of the 34 strains tested, all but three produced a cytopathic effect on human foreskin fibroblasts (HFF) and all but six strains formed large microcolonies on HFF monolayers. A subset of 12 selected strains underwent more extensive analyses and, when evaluated for both their cytadherence kinetics and growth in the presence of HFF monolayers, it was found that several of these strains had a very limited ability to attach to HFF cells. When the same 12 strains were tested in the temperature-dependent rabbit model, only the seven strains which were positive in all of these in vitro-based tests readily produced lesions. In contrast, the five strains that were noted to be deficient in one or more of the phenotypic characteristics scored in the in vitro systems did not produce lesions. This association between the traits measured in vitro and the ability to produce dermal lesions was significant ($P = 0.0012$). These results suggest that in vitro behavior may be used to predict the virulence potential of *H. ducreyi* strains. Moreover, the phenotypic characteristics described in this study are appropriate focal points for efforts to determine the molecular basis of the virulence of this pathogen.

Chancroid is a sexually transmitted ulcerogenital disease caused by the fastidious gram-negative bacterium *Haemophilus ducreyi*. In recent years, chancroid has been recognized as an important public health problem in both industrialized and underdeveloped countries (3, 22). Of particular concern, chancroid has been implicated as a cofactor in the transmission of the human immunodeficiency virus (7, 13, 18, 26, 29, 36). For these reasons, considerable effort is now being expended to define the pathogenesis of *H. ducreyi* infection at the cellular and molecular levels (1, 2, 4, 5, 9, 15, 20, 21, 30–32, 38, 39). Nevertheless, knowledge of the basic biology of this disease and its etiologic agent remains extremely limited.

Effective investigation of the virulence factors of *H. ducreyi* requires the use of appropriate model systems. In the past 3 years, both in vitro (i.e., tissue culture) and in vivo (i.e., both animal and human) models for this purpose have been described (4, 5, 9, 20, 27, 33, 37, 39). For example, human foreskin fibroblasts (HFF) have been utilized to evaluate both cytadherence (4, 5) and cellular invasion (20) by *H. ducreyi*, while HEp-2 and HeLa cells were used to detect cytotoxin production by this pathogen (19, 28). Recently, we described a temperature-dependent rabbit model for experimental chancroid which, unlike earlier animal models (14, 40), requires both a viable bacterial inoculum and bacterial replication for necrotic lesion formation (27). Our preliminary studies showed that this model can be used to evaluate the virulence of *H. ducreyi* isolates. Most recently, this model was employed to demonstrate that immunization can induce protective immunity against experimental chancroid (17).

A powerful approach to pathogenesis studies is to use genetic systems to construct isogenic mutants defective in the expression of a potential virulence factor. While the technology to generate such mutants now exists for use with cloned *H. ducreyi* genes (16), the identities of the relevant phenotypic traits remain to be determined. As a first step in addressing this issue, we investigated the correlation between selected in vitro phenotypic characteristics potentially relevant to pathogenesis and the ability of *H. ducreyi* strains to produce disease in the temperature-dependent animal model. Our objective was to identify in vitro traits that could be targeted for subsequent genetic analyses.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The 34 *H. ducreyi* strains used in this study are listed in Table 1. All of these strains have been described previously (6) and were generously provided by Allan Ronald (St. Boniface General Hospital, Winnipeg, Manitoba, Canada). A *Haemophilus influenzae* type b strain (ATCC 10211) was obtained from the American Type Culture Collection. All strains were grown on chocolate agar (CA) medium consisting of either Columbia agar base (Becton Dickinson Microbiology Systems) or GC agar base (BBL Microbiological Systems, Becton Dickinson, Cockeysville, Md.) containing hemoglobin and 0.1% IsoVitalex (BBL). The cultures were incubated at 33 to 35°C in a humidified atmosphere of 95% air–5% CO_2. Stock cultures of each strain were stored at −70°C in either fetal bovine serum or skim milk.

Inoculation of experimental animals. Male New Zealand White rabbits (2.7 to 3.2 kg) were obtained from commercial sources and housed in rooms with temperatures controlled at 15 to 17°C (27). At least 24 h before bacterial inoculation, the dorsal hair of each rabbit was shaved using an animal-grooming clipper (Model A-5; Oster Professional Products, Milwaukee, Wis.). The rabbit backs were shaved daily thereafter.

The *H. ducreyi* strains used for inoculation of animals were grown for 16 to 18 h and were harvested by scraping bacterial growth from CA plates into sterile phosphate-buffered saline (PBS), pH 7.2. Cells were washed once in PBS by centrifugation and resuspension, and serial 10-fold dilutions were made in PBS. The number of CFU of *H. ducreyi* present in the suspensions was determined by spreading 50-μl portions of each suspension on CA plates according to the procedure of Odumeru et al. (23).

The *H. ducreyi* cell suspensions in PBS were injected intradermally into the shaved backs of rabbits, using an injection volume of 0.1 ml. In every experiment, the inoculation sites were observed, palpated, and photographed at 2, 4, and 7

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days postinoculation. A numeric value was assigned to the lesions according to the method of Purcell et al. (27). This scoring system assigned the following values to clinical characteristics of the lesions: 0 = no change, 1 = erythema, 2 = induration, 3 = nodule, 4 = necrosis. For each lesion at each time point, the score was given for the most advanced characteristic (i.e., a lesion that was erythematous and indurated was assigned a value of 2 because induration is a more advanced stage). Mean lesion scores for the inoculum sizes of 10^4, 10^5, and 10^6 CFU at days 2, 4, and 7 were statistically analyzed as described below. Lesion formation by inocula greater than 10^6 CFU can be attributed to the lipooligosaccharide (endotoxin) content of the inoculum and does not require viable organisms (9, 27). Inocula smaller than 10^4 CFU fail to consistently produce necrotic lesions (27). For analysis of the virulence of *H. ducreyi* strains, at least two strains were used to infect each set of rabbits. Strain RO18 was included in every experiment and served as an internal standard for the determination of possible variation in lesion formation among experiments.

Tissue culture systems. The HFF cell line (a nontransformed cell line derived from pooled foreskins of children) was used for all attachment and cytopathic effect (CPE) studies (4). This cell line was cultivated in RPMI 1640 medium (ICN Biomedicals) supplemented with 1 mM sodium pyruvate (ICN Biomedicals), 2 mM L-glutamine (ICN Biomedicals), and 10% (vol/vol) fetal bovine serum (Gibco BRL, Grand Island, N.Y.). The cell line was subjected to at least three passages in antibiotic-free medium before use in experiments with *H. ducreyi*. The tissue culture flasks were incubated at 35°C in a humidified atmosphere of 95% air–5% CO_2. The monolayers were passaged every 72 h by using a solution of 0.05% trypsin and 0.53 mM EDTA (Gibco) to detach the cells from the plastic. Each well of a 24-well tissue culture tray (Corning Glass Works, Corning, N.Y.) was seeded with 10^5 HFF and incubated for 48 h prior to use in attachment studies. Viability determination using the trypan blue exclusion method confirmed that at least 96% of the HFF cells seeded were viable.

Attachment assay. Briefly, a cell suspension of *H. ducreyi* was prepared, and clumps of bacteria were allowed to settle. The supernatant was removed and adjusted to be equivalent to a #1 McFarland turbidity standard. Portions (10 μl) of the bacterial suspension were used to inoculate each well of the 24-well tray containing HFF cells. The number of CFU in each inoculum was determined; the multiplicity of infection was between 5 and 10 bacteria per cell. The infected monolayers were incubated at 35°C in a humidified atmosphere of 95% air–5% CO_2. At 0.5, 2, 4, and 6 h postinfection, wells were washed four times with tissue culture medium to remove unbound bacteria. The supernatant and the first wash were pooled and sonicated briefly, and then serial 10-fold dilutions were prepared. Portions (100 μl) of these dilutions were spread on CA plates which were incubated as described above to determine the number of unattached bacteria per well. To determine the number of bound bacteria, the washed monolayers then were incubated for 10 min with 300 μl of 0.05% trypsin. An additional 300 μl of tissue culture medium was then added, and the cell suspension was briefly sonicated. Tenfold serial dilutions then were prepared and spread onto CA plates as described above. Control experiments demonstrated that exposure of *H. ducreyi* to 0.05% trypsin for 10 min did not affect bacterial viability. Attachment of the bacteria to the HFF monolayer is expressed as CFU bound per well. The total CFU per well is also shown. All colony counts represent the average of triplicate wells. Interexperiment variability in the attachment assay was assessed by performing three independent attachment experiments (in triplicate) with *H. ducreyi* 35000. The use of one-way analysis of variance indicated that there was no significant difference among the means ($P = 0.995$).

Light microscopy. HFF monolayers were prepared by inoculating each well of a 96-well tissue culture tray with 10^4 HFF cells followed by incubation for 72 h at 35°C in a humidified atmosphere containing 95% air–5% CO_2. The monolayers then were infected with 10^4 to 10^5 CFU of the appropriate *H. ducreyi* strain per monolayer. An *H. influenzae* type b strain was included in these experiments as a negative control. After 24 h, the infected monolayers were washed four times with tissue culture medium to remove any unbound bacteria. The washed monolayers were stained for 20 min with crystal violet stain consisting of 0.25% (wt/vol) crystal violet, 20% (vol/vol) methanol, 0.9% (wt/vol) NaCl, and 0.02 M Tris-HCl (pH 7.5). The trays were then gently washed under running tap water until dye was no longer eluted. The monolayers were viewed by light microscopy using a Nikon Diaphot Phase Contrast-2 camera.

Statistical analyses. For each inoculum used in the rabbit model, mean lesion scores (± standard deviations) were determined on days 2, 4, and 7. Because the lesion scores were not normally distributed, all of the scores for an experiment were ranked from highest to lowest and the analyses were performed on the ranks rather than on the raw data, as recommended (10). These data were analyzed by repeated-measures analysis of variance (12), which adjusts for the correlations that arise when the same subject is measured repeatedly with respect to certain factors. In the animal experiments in this study, the repeated factors were the strain used for challenge, the day of observation, and size of the inoculum. Fisher's exact test was used to determine whether there was a significant association between a positive result in the in vitro tests and dermal lesion production in rabbits.

TABLE 1. *H. ducreyi* strains used in this study and their abilities to cause CPE in HFF and form microcolonies on HFF monolayers

Bacterial species and strain	Ability to produce CPE	Microcolony formation
H. ducreyi		
35000	+	+
54198	+	+
78226	−	−
6V	−	−
35199	−	−
A77	+	−
CIP542	+	−
36-F-2	+	−
108	+	+
HD013	+	+
HD014	+	+
MASS576	+	+
MASS579	+	+
CA173	+	+
GV072	+	+
GV073	+	+
C131	+	+
RO18	+	+
C148	+	+
BG411	+	+
181	+	+
BG407	+	+
NY006	+	+
NY012	+	+
OH1075	+	+
OR574	+	+
VII58	+	+
VII59	+	+
E1673	+	+
CH2	+	+
CH39	+	+
PU1	+	+
WPB506	+	+
WPB511	+	+
H. influenzae	−	−

RESULTS

Investigation of the interaction of *H. ducreyi* strains with HFF monolayers. Previous work with *H. ducreyi* in tissue culture systems had identified characteristics, specifically CPE, microcolony formation, and cytadherence (4, 5, 20, 28), which are believed to be relevant to bacterial virulence (11). At the outset of the present study, 34 *H. ducreyi* strains were screened for their abilities to produce CPE and form microcolonies with HFF monolayers; these particular traits can be readily assessed for a large number of strains. Only 3 (78226, 35199, and 6V) of these 34 strains failed to produce CPE (Table 1). These three CPE-negative strains, as well as an additional 3 (A77, CIP542, and 36-F-2) of the 31 CPE-positive strains, did not form microcolonies (Table 1) in the presence of the HFF cells. *Haemophilus influenzae*, which produces neither CPE nor microcolonies, served as a negative control in these experiments (Table 1).

Figure 1 contains representative micrographs of HFF monolayers 24 h after being inoculated with tissue culture medium (Fig. 1A) or with a strain of *H. influenzae* (Fig. 1D); these monolayers show no evidence of damage. In contrast, HFF monolayers inoculated with *H. ducreyi* A77 (Fig. 1B), CIP542 (Fig. 1C), RO18 (Fig. 1E), or 35000 (Fig. 1F) show considerable CPE. In addition, microcolonies formed by strain RO18 (Fig. 1E) and 35000 (Fig. 1F) were readily visible in these

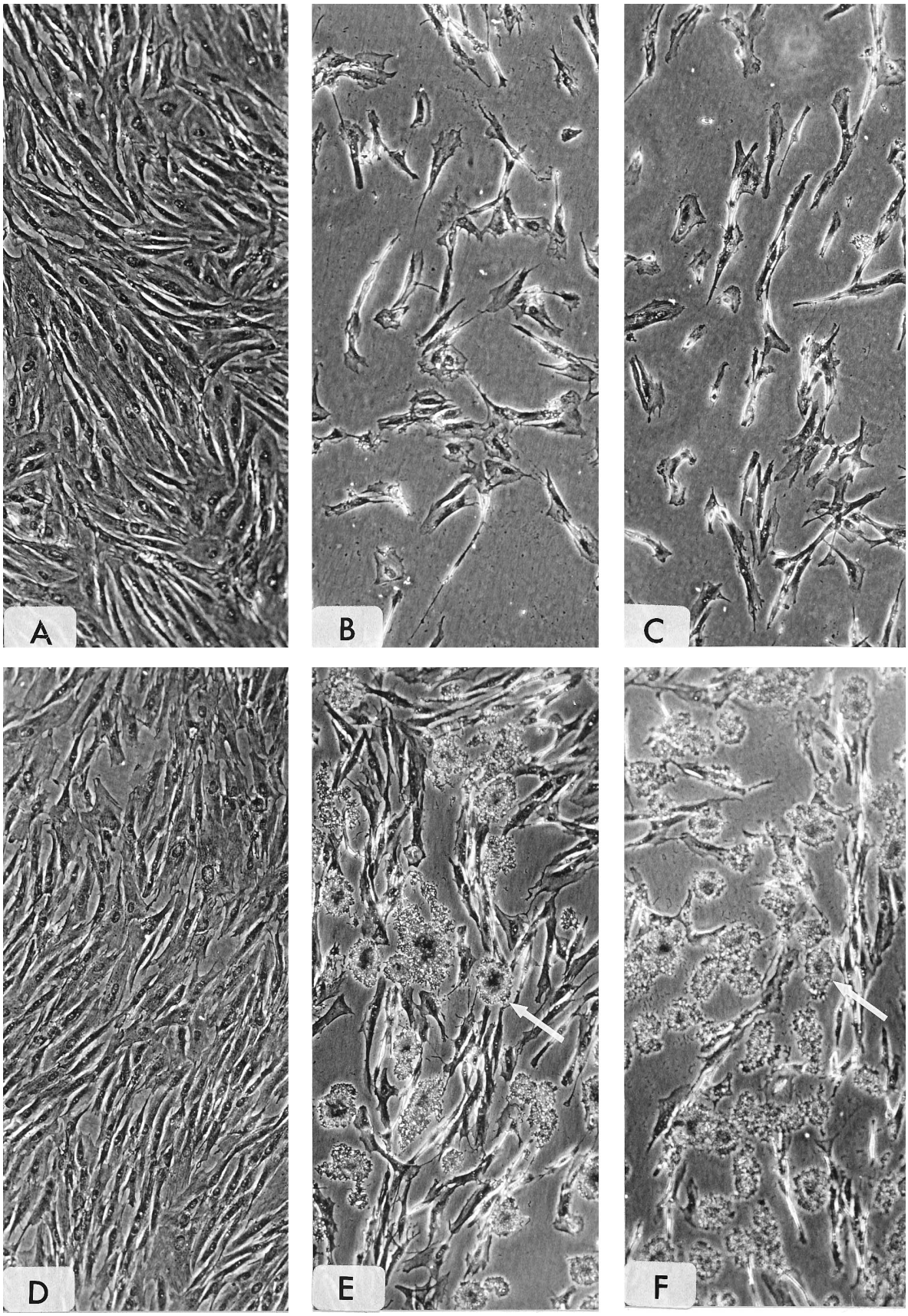

FIG. 1. CPE of *H. ducreyi* strains on HFF cells in culture. HFF cells were grown as monolayers and inoculated with tissue culture medium (no bacteria) (A) or with 10^4 CFU of *H. ducreyi* A77 (B), *H. ducreyi* CIP542 (C), *H. influenzae* type b (D), *H. ducreyi* RO18 (E), or *H. ducreyi* 35000 (F). These monolayers were incubated for 24 h and then stained with crystal violet and photographed as described in Materials and Methods. Microcolonies formed by *H. ducreyi* RO18 and 35000 are indicated by the white arrows in panels E and F, respectively.

TABLE 2. Characteristics of the selected *H. ducreyi* strains in the different model systems

Bacterial strain	CPE produced on HFF cells after 24 h	Microcolony formation on HFF cells after 24 h	Relative AI of 2.0 or greater	Dermal lesion formation in rabbit model
WPB506	+	+	+	+
181	+	+	+	+
CA173	+	+	+	+
35000	+	+	+	+
RO18	+	+	+	+
PU1	+	+	+	+
BG411	+	+	+	+
A77	+	−	−	−
6V	−	−	−	−
CIP542	+	−	+	−
78226	−	−	+	−
E1673	+	+	−	−

micrographs. No microcolony formation was evident with strains A77 (Fig. 1B) and CIP542 (Fig. 1C).

Cytadherence kinetics and growth of selected *H. ducreyi* strains in the presence of HFF monolayers. A subset of 12 of these 34 strains was subjected to more extensive analyses to identify additional phenotypic traits that might be associated with virulence expression. This strain set included two CPE-negative strains (78226 and 6V) that were also unable to form microcolonies, two strains (CIP542 and A77) that were CPE positive but unable to form microcolonies, and eight strains (35000, CA173, BG411, RO18, 181, E1673, PU1, and WPB506) that were able to produce both CPE and microcolonies (Table 2).

Two factors which can influence the degree of binding of *H. ducreyi* to HFF monolayers are the abilities of the bacterium to replicate in vitro and to attach to the human cells. Therefore, experiments were performed to determine the time course of both bacterial replication and attachment to HFF monolayers. Initially, it was found that these 12 *H. ducreyi* strains could be divided into two groups based on their ability to replicate in vitro. Three (6V, E1673, and WPB506) of the 12 strains exhibited either no net increase or a very modest increase in viable numbers over the 6-h time course of the attachment experiment (Fig. 2A depicts results obtained with strain 6V). In contrast, the other nine strains appeared to grow readily in the presence of HFF cells during this same time period (Fig. 2B to F depict results obtained with five [CIP542, A77, 35000, 181, and CA173] of these nine strains).

Determination of the AI. With both of these groups, differences were observed in the relative abilities of the strains to attach to HFF cells. To provide a more quantitative measurement of the interaction of these *H. ducreyi* strains with HFF monolayers, an attachment index (AI) was formulated to address the combined effects of growth and binding in the HFF

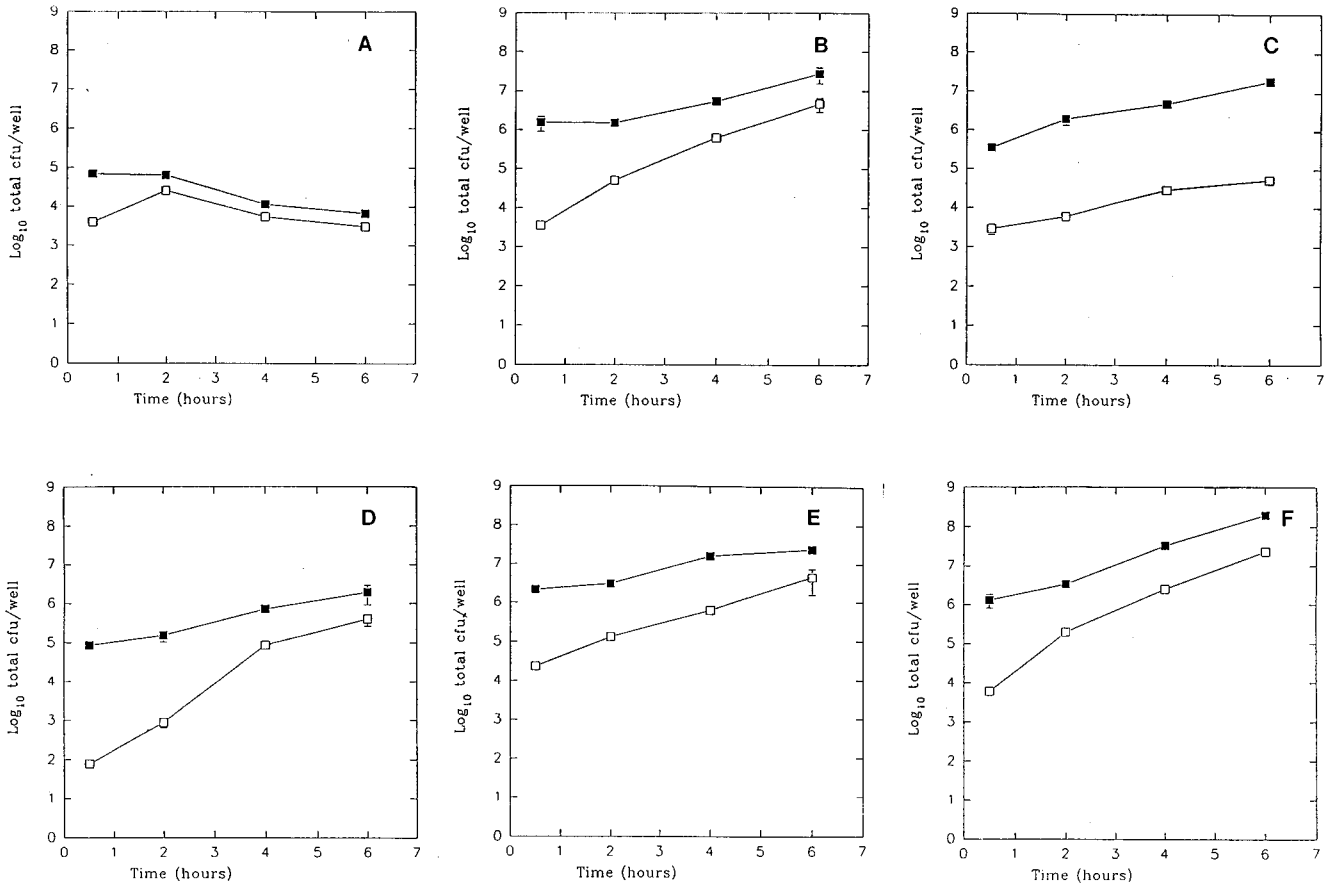

FIG. 2. Kinetics of growth and attachment of selected *H. ducreyi* strains in culture with HFF cells. HFF monolayers in tissue culture wells were infected with six *H. ducreyi* strains. The numbers of bacteria, detected as CFU, bound to the monolayer and free in the growth medium were determined as described in Materials and Methods. The results are expressed as the means of triplicate experiments; error bars indicate standard deviations. (A) 6V, (B) CIP542, (C) A77, (D) 35000, (E) 181, (F) CA173. ■, total CFU per well; □, bound CFU per well.

TABLE 3. Relative attachment and growth characteristics of the *H. ducreyi* strains used in this study

Bacterial strain[a]	Attachment factor[b] (log_{10})	Growth factor[c]	AI[d] (log_{10})	Relative AI[e]
CA173	6.62	149.1	8.79	1,219
CIP542	5.93	17.6	7.18	29.4
181	5.90	11.1	6.95	17.3
78226	5.55	24.5	6.95	17.3
BG411	5.89	11.0	6.93	16.8
35000	5.20	18.7	6.48	5.85
RO18	4.87	22.3	6.21	3.20
WPB506	5.67	4.1	6.18	2.96
PU1	4.19	68.1	6.03	2.08
A77	3.96	56.5	5.71	1.0
E1673	3.82	3.3	4.34	0.04
6V	−2.21	0.1	−1.20	−0.00003

[a] Strains are listed in order of decreasing relative AI.
[b] Attachment factor is defined as the slope of the line derived from plotting CFU bound from 0.5 to 6 h in Fig. 2.
[c] Growth factor is calculated as total CFU at 6 h divided by total CFU at 0.5 h.
[d] AI is calculated by multiplying the attachment factor by the growth factor.
[e] Relative AI is calculated by dividing the AI of a given strain by the AI of strain A77.

TABLE 4. Lesion production by selected *H. ducreyi* strains in the temperature-dependent rabbit model

Strain[a]	No. of animals infected	Mean lesion score with inoculum size (CFU/site):		
		10^6	10^5	10^4
WPB506	8	4.00 (±0)[b]	4.00 (±0)	3.33 (±0.59)
181	8	3.95 (±0.12)	3.95 (±0.12)	3.33 (±0.50)
CA173	7	4.00 (±0)	3.92 (±0.23)	3.75 (±0.46)
35000	6	3.77 (±0.17)	3.72 (±0.13)	3.44 (±0.17)
RO18	8	3.79 (±0.30)	3.40 (±0.80)	2.65 (±1.11)
PU1	8	3.25 (±0.83)	3.00 (±0.79)	2.45 (±1.06)
BG411	7	3.66 (±0.40)	2.70 (±0.41)	2.04 (±0.60)
A77	4	1.67 (±0.65)	0.66 (±0.81)	0.25 (±0.38)
6V	7	1.81 (±0.42)	0.52 (±0.32)	0.09 (±0.16)
CIP542	4	1.58 (±0.50)	0.50 (±0.43)	0.25 (±0.32)
78226	7	0.76 (±0.49)	0.33 (±0.33)	0.09 (±0.16)
E1673	8	0.79 (±0.56)	0.17 (±0.18)	0

[a] Strains are listed in order of decreasing virulence, based on the mean lesion scores derived from the use of the inoculum containing 10^5 CFU.
[b] Values are the mean lesion scores for days 2, 4, and 7 combined (± standard deviations).

monolayers. This approach was necessitated by the observation that very slow-growing strains which attached at a modest rate to the HFF monolayers had artificially inflated attachment values when the latter were calculated as the percentage of viable bacteria bound to the monolayer.

The AI was calculated by multiplying the attachment factor (the slope of the line obtained by plotting the kinetics of attachment) by the growth factor (total CFU per well at 6 h divided by the total CFU per well at 0.5 h) (Table 3). The use of the slope of the line (the attachment factor) from the attachment plot gives an indication of the rate of attachment and, because this value can be either positive or negative, it also provides an indication of an increase or decrease in the number of CFU bound to the monolayer with time (Table 3). The use of this approach allows ready distinction between strains with slow attachment kinetics (e.g., A77 [Fig. 2C]) and those which attach rapidly to HFF cells (e.g., CIP542 [Fig. 2B]). In contrast, the growth factor provided an indication of the ability of an individual *H. ducreyi* strain to grow in the presence of HFF monolayers (Table 3). A growth factor of 10 indicated that the recoverable CFU per well for the strain had increased by 10-fold over the 6-h experimental period, whereas a growth factor of 0.1 indicated a 10-fold decrease in viable organisms over the time course of the experiment.

For the purpose of comparing the abilities of various strains to attach to HFF cells, the AI of each strain was divided by that of the attachment-defective strain A77 to yield a relative AI (Table 3). Strain A77 was chosen for this purpose because it has been well characterized in previous studies (4, 5) and grows well in the HFF system (Fig. 2C) but attaches very poorly to these human cells (i.e., less than 1% of the A77 organisms present at the 6-h time point were attached to HFF cells [Fig. 2C]). Therefore, A77, with a relative AI of 1, is representative of an attachment-deficient strain within the context of this in vitro model. In the present study, *H. ducreyi* strains that exhibited a relative AI of 2 or greater were considered to be attachment positive. Those with a relative AI of less than or equal to 1 were defined as being attachment deficient in the HFF system.

By use of relative AI values to compare these 12 strains, again strains 6V and E1673 had the lowest scores in this assessment of cytadherence ability (Table 3). For strain 6V, this was primarily the result of an inability to replicate in the HFF system (growth factor of 0.1). With strain E1673, the low relative AI value was caused by a combination of a slow rate of growth (growth factor of 3.3) and a very poor attachment ability (attachment factor of [log_{10}] 3.82). The other nine strains all had relative AI values of 2 or greater, indicating that they possessed the abilities to replicate and attach well in the HFF system over the 6-h experimental period.

For the purpose of comparison, the phenotypic characteristics of these 12 *H. ducreyi* strains in these in vitro systems are summarized in Table 2. These traits include their abilities to produce CPE on HFF monolayers, to form microcolonies in the presence of HFF cells, and to attach to the same human cells with a relative AI of at least 2.0.

Comparison of lesion production by selected *H. ducreyi* strains. The 12 strains described above were next evaluated in the temperature-dependent rabbit model to determine whether any correlation existed between their in vitro phenotypic characteristics (listed in Table 2) and their abilities to produce dermal lesions. Strain RO18 was included in every experiment to provide an internal standard for lesion development. When the mean lesion scores obtained with strain RO18 were analyzed for interexperiment variation, no significant difference was detected among five experiments involving a total of 40 rabbits ($P = 0.099$).

Previously, an *H. ducreyi* strain was considered to be virulent in this model if it produced nodular or necrotic lesions with an inoculum of 10^5 CFU (27). By this criterion, 6 of the 12 strains were virulent, yielding mean lesion scores of at least 3.00 from an inoculum of 10^5 CFU (Table 4). Figure 3A shows the lesions obtained with two of these strains. Strain 35000, an isolate from Winnipeg, Manitoba, Canada, which has been well characterized in both tissue culture models (4, 5) and animal models (9, 14, 17, 27), formed lesions equivalent to those produced by RO18, a recent isolate from Kenya. A seventh strain (BG411) formed lesions that were either indurated or nodular with an inoculum of 10^5 CFU. Necrotic lesions, however, were seen with a 10-fold-greater inoculum of this strain (Table 4). It should be noted that all seven of these strains were positive in all of the in vitro tests (Table 2).

In contrast, the remaining five *H. ducreyi* strains barely produced erythema with an inoculum of 10^5 CFU (Table 4). Even

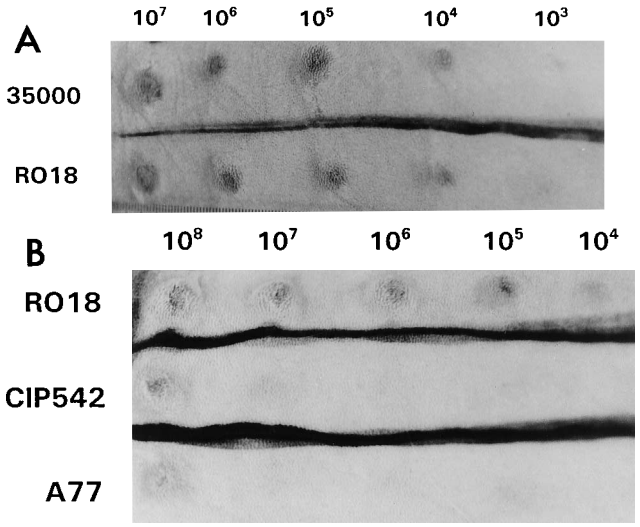

FIG. 3. Dermal lesions produced in the temperature-dependent rabbit model by selected *H. ducreyi* strains. Equivalent numbers of viable cells of the four *H. ducreyi* strains were injected intradermally into the backs of rabbits housed at 15 to 17°C; the inoculum sizes are listed above each set of sites. Lesion development is shown at 4 days after inoculation. (A) Strains 35000 and RO18; (B) strains RO18, A77, and CIP542.

with a 10-fold-greater inoculum, these five strains induced only minimal lesions (Table 4). Figure 3B shows the very modest lesions obtained with two of these strains, A77 and CIP542, in comparison to the lesions produced by the virulent strain RO18. It also must be noted that each of these five relatively avirulent strains was deficient in one or more of the in vitro characteristics listed in Table 2. The association between a positive result from the in vitro tests and the ability to readily produce dermal lesions in rabbits was significant ($P = 0.0012$).

DISCUSSION

In recent years, numerous laboratories have attempted to develop potentially relevant in vitro and in vivo systems that can be used to begin to elucidate the mechanisms by which *H. ducreyi* causes genital ulcers. Using these systems, investigators have identified phenotypic characteristics of various isolates of this bacterium that reasonably might be expected to correlate with virulence expression (4, 5, 19, 22, 28, 39). Nevertheless, a clear definition of virulence for this pathogen has not been established. To begin to address this issue, we evaluated 34 *H. ducreyi* isolates in a well-characterized tissue culture model (4, 5, 27) and then tested the virulence of a dozen of these strains in the temperature-dependent rabbit model (27). To the best of our knowledge, this is the first time that phenotypic traits identified for *H. ducreyi* in a tissue culture system have been correlated with virulence expression in an animal model.

One advantage of in vitro systems relative to animal models is that they permit dissection, at the molecular level, of specific, individual phenotypic traits of pathogens. In this study, HFF monolayers proved to be useful in evaluating not only the relative attachment capabilities of several *H. ducreyi* strains but also their abilities to replicate in vitro, to form monolayer-adherent microcolonies, and to produce CPE. This study revealed that 3 of these 34 strains were unable to produce CPE (Table 1). These three strains (78226, 35199, and 6V) also lacked the ability to form microcolonies, although this same defect was also present in three other strains (A77, CIP542, and 36-F-2) that did produce CPE. The ability to produce CPE in HFF monolayers was, therefore, apparently independent of the ability of these strains to form microcolonies. The cohesiveness of cells of fresh clinical isolates of *H. ducreyi* is a well-recognized phenotypic characteristic of this organism (3, 22, 35) and is likely responsible for the microcolony formation observed in both this and a previous study (4). The potential importance of microcolony formation is reinforced by their presence in lesions formed after intradermal inoculation of rabbits with the virulent 35000 strain (27).

These in vitro tests also revealed that attachment of *H. ducreyi* to the HFF monolayers was not a prerequisite for production of CPE because at least two strains (A77 and E1673) that were CPE positive were essentially unable to attach to the HFF cells (Tables 2 and 3). In fact, strain E1673 was barely able to survive in culture with HFF cells (Table 3) and yet it still produced CPE. In addition, the ability to attach to HFF cells was apparently independent of microcolony formation, as the attachment-positive strains 78226 and CIP542 did not form microcolonies (Tables 1 and 2). It should be noted that strain E1673, which did form microcolonies, had a very low relative AI (0.04), which was the result of both its slow growth in vitro and its very poor attachment to HFF cells (Table 3).

There are several caveats associated with the experiments described above which involved the study of *H. ducreyi* growth rates and kinetics of adherence to HFF cells in vitro. First, the enumeration of viable organisms (i.e., CFU determinations) to measure the rate of increase in bacterial numbers could be artificially skewed by the tendency of *H. ducreyi* to autoaggregate (3, 22), and strain-to-strain variability in this trait could have affected these numbers. Second, variability in autoaggregation among *H. ducreyi* strains could have artificially increased the number of bacteria that came into contact with the HFF cells in the attachment assays (i.e., large clumps would settle more quickly and yield a higher degree of attachment). To minimize this possibility, the smallest possible volume of tissue culture medium was used to cover the HFF monolayer, thus ensuring maximal interaction between the suspended bacteria and the monolayer. Finally, large clumps of bacteria were removed from the original suspension prior to its use as the inoculum for the HFF monolayers. We cannot eliminate the possibility that the bacteria in these aggregates (which were removed from the suspension) had different adherence characteristics (for HFF cells) from those of the bacteria that were actually introduced into the adherence assays.

We previously demonstrated that the temperature-dependent rabbit model could be used to distinguish the relative virulence of *H. ducreyi* strains (27). In that study (27), we described a clinical isolate (strain 041) that consistently required a larger inoculum than did strain 35000 to produce necrotic lesions. In the present study, 5 of 12 *H. ducreyi* strains tested in the same animal model proved to be relatively avirulent (Tables 2 and 4). While several interesting correlations were apparent among the in vitro phenotypic characteristics of these *H. ducreyi* strains, perhaps the most significant finding was the fact that all five of the relatively avirulent strains were defective in at least one of the characteristics measured in the HFF-based systems (Table 2). One (CIP542) of these relatively avirulent strains lacked only the ability to form microcolonies, while another (E1673) was deficient primarily in its ability to attach to HFF cells (Table 2). The other relatively avirulent strains (78226, 6V, and A77) lacked at least two of the phenotypic traits examined in this study (Table 2). In contrast, the seven strains which were virulent in the animal model were also positive in all of the HFF-based in vitro tests (Table 2).

Our results from these animal studies also corroborate the

findings of Hammond et al. (14), who found that both A77 and CIP542 were less virulent than strain 35000 in an animal model that did not involve reduced ambient temperatures. These earlier studies, which included the discovery that serum resistance of this pathogen was associated with expression of a certain type of lipooligosaccharide (24, 25), were the first to delineate potential virulence markers for *H. ducreyi.*

Recently, it was reported that many strains of *H. ducreyi* express cytotoxic activity (19, 28). While we cannot confirm that the CPE measured in the present study is mediated by the same microbial product(s) as that reported by Lagergard and Purven (19, 28), lesion formation in the temperature-dependent rabbit model does not appear to be due solely to those *H. ducreyi* components that promote damage of HFF monolayers. Lagergard (19) also noted that different *H. ducreyi* strains can produce lesions in rabbits regardless of their expression of the putative cytotoxin. From these results, she concluded that the failure of the rabbit model to reflect the cytotoxic activities of different *H. ducreyi* strains limited the animal model's usefulness for pathogenesis studies. However, the animals used in the latter studies were housed at 18 to 20°C (19), and we have noted that even transient increases in ambient temperature above 17°C have deleterious effects on lesion production and significantly increase the size of inoculum required for lesion formation. The findings of the present study, in which three CPE-positive strains (A77, CIP542, and E1673) proved to be relatively avirulent, suggest the alternative interpretation that production of CPE, at least as measured with HFF monolayers, does not by itself indicate the potential of various *H. ducreyi* strains to form lesions in an animal model.

One serious limitation to the use of strains such as CIP542, A77, and 78226 as representative avirulent strains is that the genetic defect(s) responsible for their apparent avirulence is not known. Similarly, there are no data available concerning the extent of genetic relatedness among either these strains or the virulent strains included in this study. At present, investigations of the type described in this report, involving differences between virulent and avirulent *H. ducreyi* strains, have been limited to the use of such poorly defined avirulent strains. Mutagenesis techniques would make it possible not only to identify the genetic loci responsible for phenotypic traits such as attachment and microcolony formation but also to prove unequivocally the relationship between these traits, lesion formation in the animal model, and virulence of *H. ducreyi*. Fortunately, preliminary reports of generalized mutagenesis systems for use with *H. ducreyi* have appeared recently (8, 34). It should now be possible to begin constructing truly isogenic mutants of *H. ducreyi*, deficient in their abilities to express the various in vitro phenotypes examined in this study, and to test these mutants for their virulence potential in vivo.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Manitoba Health Research Council to M.J.A. and by Public Health Service grant AI32011 to E.J.H. and J.D.R.

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