Potential Virulence-Associated Factors in Brazilian Purpuric Fever

GEORGE M. CARLONE,1,6 LEO Gorelkin,1 LINDA L. GHEESLING,1 ALICE L. ERWIN,2 SUSAN K. HOISETH,3 MARTHA H. MULKS,2 STEVEN P. O’CONNOR,1 ROBBIN S. WEYANT,3 JAMES MYRICK,1 LORRY RUBIN,6 ROBERT S. MUNFORD III,2 ELIZABETH H. WHITE,1 ROBERT J. ARKO,1 B. SWAINMATHAN,1 LEWIS M. GRAVES,1 LEONARD W. MAYER,3 MARY K. ROBINSON,1 SAMUEL P. CAUDILL,1 AND THE BRAZILIAN PURPURIC FEVER STUDY GROUP†

Centers for Disease Control, Atlanta, Georgia 30333; Division of Infectious Diseases, Department of Internal Medicine, The University of Texas Health Sciences Center, Dallas, Texas 75206; Department of Microbiology, Georgetown University, Washington, D.C. 20057; Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824; Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia 30322; and Schneider Children’s Hospital of Long Island Jewish Medical Center, New Hyde Park, New York 11040.

INTRODUCTION

Haemophilus influenzae biogroup aegyptius (H. aegyptius) is the etiologic agent of a newly recognized disease, Brazilian purpuric fever (BPF) (5–7). A single H. influenzae biogroup aegyptius clone, the characteristics of which define the case clone phenotype (7), hereafter called the BPF clone, is responsible for all of the cases of BPF analyzed in Brazil. While the histopathology of BPF has been described (5), the virulence factors associated with H. influenzae biogroup aegyptius are undefined. Attempts have been made, however, to correlate specific H. influenzae biogroup aegyptius phenotypic characteristics with BPF and to establish in vivo models. This review describes several potential Haemophilus virulence factors (27), including lipooligosaccharides (LOS), capsular polysaccharides, pilus proteins, immunoglobulin A1 (IgA1) proteases, membrane-associated proteins, and extracellular proteins to determine their potential role in BPF. In addition, two in vivo systems, a chicken embryo infection model (35) and the infant rat bacteremia model (36), were evaluated as tools to study pathogenesis associated with H. influenzae biogroup aegyptius. Information presented at the Brazilian Purpuric Fever Workshop held at the Centers for Disease Control, Atlanta, Ga., on 13 to 14 May 1988, was abstracted for this review.

HISTOPATHOLOGY

Histopathologic examination of tissues from six fatal cases of BPF (three epidemic and three sporadic) has shown hemorrhage, intravascular microthrombi, and necrosis (5). Hemorrhage was present in the skin, lungs, and adrenal glands, often particularly extensive in the latter two organs. Intravascular microthrombi were present in the upper dermis, renal glomeruli, lungs, and hepatic sinusoids. Acute necrosis was variable and focal in lymphatic organs, such as the germinal centers of lymph nodes and Peyers’ patches, and in splenic white pulp. Less consistently present were evidence of acute respiratory distress with alveolar hyaline membranes and pulmonary edema. No evidence of vasculitis or immunopathologic phenomena was found by light microscopy, nor were other specific or significant histopathologic changes seen, such as viral inclusions or meningitis.

The histopathologic evidence is consistent with acute shock syndrome mediated by some form of consumption coagulopathy characterized by focal intravascular coagulation and consequent hemorrhagic diathesis. These findings are characteristic of purpura fulminans (37), a well-described sequela of various viral and bacterial infections. High levels of endotoxin (mean, 675 pg/ml; range, 100 to 1,600 pg/ml), as determined by a quantitative Limulus amoeocyte lysate assay, were detected in eight cases of BPF (5). Although various factors and mechanisms can initiate such terminal common pathophysiologic pathways, endotoxia can produce this clinical picture in humans. Once established, endotoxia and its effects would be enhanced by a self-perpetuating cycle via reticuloendothelial depression, as seen in BPF, exemplified by lymphatic necrosis and hepatic sinusoidal saturation by fibrin.

LOS GEL ELECTROPHORESIS PROFILES AND IMMUNOREACTIVITY

Surface-exposed components of Haemophilus species, which include LOS, protein, and capsular polysaccharide, have been associated with both virulence and immunity (27). The structure, immunoreactivity, and cytotoxicity of H. influenzae biogroup aegyptius LOS, possibly the effector of the terminal events in BPF, have been examined. Seven distinct LOS profiles (I to VII) were visualized by silver staining after sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis (SDS-urea-PAGE) of 40 proteinase K-treated whole-cell suspensions (14, 39). LOS with profiles I, II, and III migrated into a single band, LOS with profiles IV, V, and VI migrated into double bands, and LOS with profile VII migrated into triple bands. Of 40 isolates tested, isolates of the BPF clone were restricted to LOS profiles of I (11 of 12 strains), II (4 of 6 strains), or IV (10 of 10 strains). Two of the three strains in these profiles that were not BPF clone...
strains agglutinated with rabbit polyclonal antiserum to the BPF clone reference (7).

The strains were further divided into eight (1 to 8) monoclonal antibody (MAB) reactivity patterns after SDS-urea-PAGE and Western blot (immunoblot) analysis (38) by using two MABs to *H. influenzae* biogroup aegyptius LOS and six against *H. influenzae* LOS. All isolates of the BPF clone had at least one of the following: (i) an LOS profile of I and a MAB reactivity pattern of 5 \( (n = 11) \); (ii) an LOS profile of IV and a MAB reactivity pattern of 1 \( (n = 10) \); (iii) an LOS profile of II and a MAB reactivity pattern of 1 \( (n = 3) \) or 8 \( (n = 1) \). A control strain of *H. influenzae* serotype b had an LOS profile and a MAB reactivity pattern different from that of any *H. influenzae* biogroup aegyptius strain. The LOS results for *H. influenzae* biogroup aegyptius suggest that a restricted number of LOS structures is associated with invasive disease.

**INFECTION OF CHICKEN EMBRYOS**

Since the histopathology of BPF suggested that bacterial LOS plays a role in pathogenesis, a chicken embryo infection model was examined. Chicken embryos are extremely sensitive to LOS, with a 50% lethal dose \( (LD_{50}) \) of about 10 ng (10). Pittman and Davis (35) showed that 7- to 12-day-old chicken embryos are susceptible to experimental infection by *H. influenzae* biogroup aegyptius. Chicken embryos of various ages were infected via several routes (4, 13) with two strains of *H. influenzae* biogroup aegyptius. One strain was the BPF clone (with an LOS profile of IV and a MAB reactivity pattern of 1), and the other had a non-BPF clone phenotype (with an LOS profile of III and a MAB reactivity pattern of 5).

Following inoculation with either *H. influenzae* biogroup aegyptius strain, infected embryos died within about 3 days. When embryos were killed and cultured within 2 days of infection, over \( 10^6 \) CFU of *H. influenzae* biogroup aegyptius per ml of allantoic fluid were recovered; embryos that died also had more than \( 10^6 \) CFU of *H. influenzae* biogroup aegyptius per ml. When embryos surviving 4 days or longer were cultured, few or no bacteria were recovered, even from eggs inoculated with \( 10^8 \) or \( 10^9 \) CFU. No significant differences were observed between the BPF clone and the non-BPF clone isolates.

Additional experiments were conducted in which the age of the embryo and the route of inoculation were varied. Seven-day-old embryos were very susceptible to infection via the amniotic sac, with \( LD_{50}s \) of 110 and 17 CFU for the non-BPF clone isolate and the BPF clone isolate, respectively; the results for yolk sac injection were very similar. Infection of 9- to 12-day-old embryos via less invasive routes (inoculation onto the chorioallantoic membrane or into the allantoic sac) gave much higher \( LD_{50}s \), usually greater than \( 10^2 \) but in some cases over \( 10^5 \) CFU. In most experiments, the \( LD_{50} \) for the BPF clone isolate was slightly lower than that for the non-BPF clone isolate, but the ratio of \( LD_{50}s \) for the two strains rarely exceeded \( 10^2 \). Conditions that consistently demonstrated a substantial increase in virulence for the BPF clone isolate were not found. This may be attributable to many potential variables, including the following: the small number of strains tested, lack of cell binding due to the requirement for a species-specific ligand, an unnatural host and site of infection, the lack of recent animal passage of the infecting organism, and suboptimal bacterial growth and egg incubation conditions.

**SURFACE-EXPOSED CARBOHYDRATE-REACTIVE MATERIAL**

*H. influenzae* capsular polysaccharide has antiphagocytic properties and contributes to the virulence associated with this organism. BPF clone isolates of *H. influenzae* biogroup aegyptius appeared to have a capsule when stained with ruthenium red (carbohydrate-reactive material) and examined, after thin sectioning, by transmission electron microscopy (E. H. White, G. M. Carlone, and the Brazilian Purpuric Fever Study Group, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, C-8, p. 329). This carbohydrate-reactive material was observed on BPF clone isolates only after the cells were passaged in mice with subcutaneous chamber implants. The amount of carbohydrate-reactive material on BPF clone isolates was significantly less than that observed with an *H. influenzae* serotype b control strain; no reactive material was observed on non-BPF clone isolates. Less than 50% of the bacteria per field had detectable amounts of carbohydrate-reactive material. The methods used for cell growth greatly affected the amount of observable reactive material. What appear to be capsules on *H. influenzae* biogroup aegyptius strains in the bone marrow of BPF cases have been reported (31). Attempts to isolate an acidic capsular polysaccharide with Cetavlon (17) after in vitro growth were unsuccessful. Studies are in progress to purify the carbohydrate-reactive material and to confirm the presence of capsules on BPF clone isolates of *H. influenzae* biogroup aegyptius.

**HAEMOPHILUS CAPSULAR GENE PROBE**

Chromosomal DNAs isolated from BPF clone and non-BPF clone isolates of *H. influenzae* biogroup aegyptius were analyzed by Southern hybridization to a 2.1-kilobase (kb) DNA probe containing sequences known to be necessary for *H. influenzae* serotype b capsule expression (15, 16). The sequences contained in this probe are thought to be common to all encapsulated *H. influenzae* but appear to be absent in most nonencapsulated strains (15). The 2.1-kb probe was derived from a 9-kb *EcoRI* fragment of chromosomal DNA described previously (15) and contains a portion of the *capB* locus thought to be necessary for export of capsular polysaccharide (15, 21). Eleven of 11 BPF clone isolates had a 10.2-kb *EcoRI* fragment homologous to this probe, whereas none of 8 non-BPF clone *H. influenzae* biogroup aegyptius isolates exhibited homology to the probe (non-BPF clone isolates included representatives of six different isoenzyme types [7] and were from Brazil, Texas, and Egypt). The actual portion of the 2.1-kb probe responsible for hybridization of the BPF isolates has not been defined, and it is not known whether hybridization is due to coding or noncoding regions of the *capB* probe. Whether hybridization of the BPF strains to the 2.1-kb probe is simply another marker for the BPF clone or whether it is important to the virulence of the BPF strains remains to be determined.

**IgA1 PROTEASE PHENOTYPE AND GENOTYPE**

IgA1 proteases are extracellular enzymes produced by several species of bacteria that infect human mucosal epithelial surfaces, including *H. influenzae*, *Neisseria meningitidis*, *N. gonorrhoeae*, and *Streptococcus pneumoniae* (29). These enzymes are specific for human IgA of subclass 1; each enzyme cleaves a single peptide bond in the IgA1 hinge region to yield intact Fab and Fc fragments. Several
bacterial species produce more than one type of IgA1 protease, although in general a given isolate can make only one enzyme type.

IgA1 proteases of three distinct specificities have been demonstrated in *H. influenzae* (29, 30). *Haemophilus* IgA1 protease type 1 cleaves a prolyl-seryl bond at position 231 to 232 in the hinge region of the alpha chain of human IgA1. *Haemophilus* IgA1 protease type 2 cleaves a prolyl-threonyl bond between residues 235 and 236. The exact position of the peptide bond cleaved by the *Haemophilus* type 3 protease is not known. Excellent correlation between the type IgA1 protease produced and the capsular serotype has been observed in *H. influenzae*. Serotypes a, b, d, and f generally produce type 1 protease, while serotypes c and e produce the type 2 enzyme (29, 30). In a previous study of *H. influenzae* biogroup aegyptius, all nine conjunctival isolates produced type 1 IgA1 protease (19).

Phenotypic characterization of the IgA1 protease type was done by SDS-PAGE analysis of IgA1 cleavage products and assignment of protease types by comparison with controls of known protease types (30). All 15 non-BPF clone isolates (isolates from patients with conjunctivitis in the United States and Western Europe) and 21 non-BPF clone isolates isolated from patients with conjunctivitis in Brazil produced IgA1 protease type 1. All 15 BPF clone isolates produced IgA1 protease type 2, but the isolate from Australia (24), which is not included in the BPF clone, produced IgA1 protease type 1.

Restriction site polymorphism in the IgA1 protease gene (iga) was studied by using a cloned type 1 protease gene from *H. influenzae* serotype d (8) as a probe to examine Southern hybridization of genomic DNA purified from BPF clone and non-BPF clone *H. influenzae* biogroup aegyptius isolates. Preliminary results suggest that the BPF clone isolates exhibit similar and unique iga restriction patterns (i.e., the same iga genotype) not found in non-BPF clone *H. influenzae* biogroup aegyptius isolates. Comparison of iga genes from BPF clone isolates with genes from *H. influenzae* serotypes a to f, particularly with the genes encoding type 2 enzymes from serotypes c and f, are in progress.

IgA1 protease analysis and studies of restriction site polymorphism of the iga gene suggest that BPF clone isolates are clonally derived. BPF clone isolates produce an IgA1 protease that is phenotypically distinct from that produced by other *H. influenzae* biogroup aegyptius isolates. These results match well with other data demonstrating the clonal nature of the BPF clone isolates (7). Whether the IgA1 protease type is a virulence factor in BPF remains to be determined.

**EXTRACELLULAR PROTEIN**

With the exception of IgA protease, extracellular proteins that may contribute to the virulence of *Haemophilus* species have not been well characterized. A survey of *H. influenzae* biogroup aegyptius strains was undertaken to examine proteins secreted into culture supernatants. Proteins in culture supernatants, concentrated 10- to 15-fold by ammonium sulfate precipitation, were analyzed by electrophoresis on discontinuous SDS-PAGE gels (22). The proteins were visualized by silver staining (26).

A total of 169 *H. influenzae* biogroup aegyptius isolates were examined. A protein with an estimated molecular mass of 38 kilodaltons (kDa) was detected in the supernatants of all 58 BPF clone isolates. On Western blots, this protein reacted with polyclonal rabbit serum to whole cells of strain F3031. Of 105 non-BPF clone isolates, 98 did not produce a 38-kDa protein. The remaining 7 strains produced a 38-kDa protein that did not, however, react with anti-F3031 serum. An additional non-BPF clone isolate, isolated from a patient with BPF in Alice Springs, Australia (24), also produced a 38-kDa protein that failed to react with anti-F3031 serum. These results indicate that the 38-kDa proteins produced by these eight strains, although of the same apparent molecular mass, are immunologically different from the 38-kDa protein produced by BPF clone isolates.

The inability to detect the 38-kDa protein in total cellular protein and outer-membrane protein preparations suggests that it is secreted. This protein is not heat modifiable, does not form intermolecular disulfide bonds (determined by SDS-PAGE in the presence or absence of 2-mercaptoethanol), and appears to be fairly hydrophilic. Extended storage in solution at 4°C, followed by size exclusion chromatography, revealed that aggregates of the 38-kDa protein are not formed.

While the 38-kDa protein is highly correlated with *H. influenzae* biogroup aegyptius strains that have demonstrated the capability to cause BPF (the sole exception being the Australian BPF isolate), it is unknown whether this protein plays a role in the pathogenesis of BPF. As an extracellular protein, one could speculate that the 38-kDa protein functions as an exotoxin having activity that plays a role in the pathogenesis of BPF.

**PILIN PROTEIN**

An *H. influenzae* biogroup aegyptius 25-kDa protein that has been identified by SDS-PAGE (22) shares many biochemical and immunologic characteristics with an *H. influenzae* serotype b pilin described by Guerina et al. (12). This protein was found in 63% (39 of 63) of BPF clone isolates and 4% (3 of 84) of non-BPF clone isolates of Brazilian origin.

Fractionation of a BPF clone isolate by lithium chloride extraction (23) yielded outer-membrane vesicles that contained the 25-kDa protein. The protein was insoluble in most detergents, including sarcosyl and β-octylglucoside, and required heating to greater than 80°C for solubilization in SDS. When membrane associated, the 25-kDa protein was resistant to cleavage by most proteases, including trypsin, chymotrypsin, proteinase lys-C, and protease arg-C, but was digested by thermolysin and proteinase K. After solubilization, however, the protein was susceptible to digestion by all of these enzymes. The 25-kDa protein exists in a large (>200-kDa) heat-labile complex that is reactive with rabbit anti-25-kDa serum and with MAbs specific for BPF strains. Disruption of this complex by heating or proteolysis essentially eliminated binding by BPF-specific MAbs.

The N-terminal sequence of the 25-kDa protein from amino acids 3 through 20 is identical to the corresponding *H. influenzae* serotype b pilin sequence (12), with the exception of amino acid 4, which is alanine in the 25-kDa protein and threonine in the *H. influenzae* pilin. Amino acid composition analysis of the 25-kDa protein showed a high proportion of hydrophobic amino acids (leucine + isoleucine + valine + methionine = 26%) and a lysine content of 10.5%. This composition is similar but not identical to that reported for the *H. influenzae* pilin (12).

The potential for the 25-kDa protein to act as an agglutinin was assessed by immunizing a rabbit with membrane vesicles containing the 25-kDa protein and testing the immune serum for agglutination with 25-kDa-positive and -negative *H. influenzae* biogroup aegyptius strains. Of 91
strains tested, 100% (26 of 26; 23 BPF clone isolates) of the 25-kDa-positive strains agglutinated; conversely, 100% (65 of 65; 20 BPF clone isolates) of the 25-kDa-negative strains failed to agglutinate. These results were compared with agglutination results obtained with antiserum to whole-cell BPF clone isolates, and 50% (29 of 58) of the strains that agglutinated in whole-cell antiserum failed to agglutinate in anti-25-kDa protein serum. These findings suggest that the 25-kDa protein is one of multiple agglutinogens present on the surface of these strains.

The role of pilin in *H. influenzae* serotype b disease has been studied (34; M. M. Farley, D. S. Stephens, S. L. Kaplan, and E. O. Mason, Jr., Clin. Res. 36:20A, 1988). The results of these studies suggest that pilin mediates adherence to nasopharyngeal mucosa but not invasion of these tissues. We are attempting to determine the role of the 25-kDa protein as a pilus subunit and its relationship to BPF pathogenesis.

**TWO-DIMENSIONAL PROTEIN GEL ELECTROPHORESIS**

*H. influenzae* biogroup aegyptius isolates (*n* = 9) of the BPF clone isolated from the skin, blood, or bloody hemorraghic cerebrospinal fluid of patients with BPF and non-BPF clone isolates from the eyes of patients with conjunctivitis were analyzed by high-resolution two-dimensional electrophoresis (1, 2, 33). Calibration of the gels for *M*. was done with a rat heart homogenate (11), and calibration for relative charge was done with carbamylated glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (3).

Detergent-solubilized and sonicated whole-cell proteins were separated by two-dimensional electrophoresis and silver stained (32). The stained proteins were scanned, quantified, and matched by computer imaging by a Visage 2000 system. Automatic global matching of all samples to a BPF clone reference sample was done in addition to matching to a reference non-BPF clone sample from Brazil. The spot-matching software returned 4.7 and 7.6% missed matches between the BPF clone reference isolate and the non-BPF isolates, respectively, and 1.5 and 3.7% bad matches between the BPF clone reference isolate and the non-BPF clone isolates, respectively, based on 1,341 resolved protein spots in the BPF clone reference sample.

A particular Boolean expression yielded three proteins that were unique to BPF clone isolates: (i) 121.3 kDa, −22.00 G3PDH; (ii) 28.2 kDa, −17.16 G3PDH; and (iii) 14.0 kDa, −18.37 G3PDH. The more negative charge values represent the more acidic proteins. With a less restrictive Boolean expression, 90 proteins were identified in at least one BPF clone isolate but were absent in all non-BPF clone isolates, and 20 had intensities of >0.1 U in each gel in which they were detected. Similarly, 228 proteins were found in at least one non-BPF clone isolate but were absent in all BPF clone isolates, and 93 had intensities of >0.1 U.

An exploratory analysis based on Bonferroni-adjusted (25) two-group comparisons of the mean intensities of all matched proteins found three proteins with significant intensity differences between BPF clone and non-BPF clone isolates: (i) 30.0 kDa, −21.20 G3PDH; (ii) 14.4 kDa, −18.33 G3PDH; and (iii) 29.0 kDa, −25.82 G3PDH.

Studies are in progress to separate the three BPF clone isolate proteins by two-dimensional electrophoresis, electrotransfer them to a Teflon matrix, and perform partial amino acid sequence analysis to generate synthetic oligonucleotide probes for diagnostic purposes.

**INFANT RAT BACTEREMIA MODEL**

An infant rat model of bacteremia (36) was implemented with BPF clone and non-BPF clone *H. influenzae* biogroup aegyptius isolates to develop a tool for studies of pathogenesis, bacterial virulence determinants, and host defense against infection. Infant rats have previously proven useful for studying bacteremia and meningitis due to *H. influenzae* serotype b (28, 36). Following intraperitoneal inoculation with *H. influenzae* biogroup aegyptius, 66% (*n* = 30) of complement-depleted (treated with cobra venom factor [9]) 6-day-old rats had detectable bacteremia following inoculation with 10⁴ CFU of one of three BPF clone isolates, compared with only 3% (*n* = 26) of rats injected with a similar inoculum of one of three non-BPF clone isolates. An animal-passaged BPF clone isolate regularly caused bacteremia in normal 6-day-old rats. Seventy-one percent (*n* = 33) of rats injected intraperitoneally with 10³ to 10⁴ CFU developed bacteremia; greater than 90% of rats injected with 10⁵ CFU of these strains developed bacteremia. The magnitude of bacteremia was 10³ to 10⁴ CFU/ml, and meningitis was frequently present in bacteremic rats. Bacteremia was spontaneously cleared within 5 days.

Immune serum was obtained following three weekly injections of whole bacteria of a BPF clone isolate into adult rats. When this serum was given to normal 6-day-old rats subcutaneously 4 h before intraperitoneal injections with 10⁷ CFU of an animal-passaged BPF clone isolate, none of 33 rats had detectable bacteremia, in contrast to 22 (88%) of 25 rats pretreated with normal adult rat serum. Immune rat serum to a non-BPF clone isolate of *H. influenzae* biogroup aegyptius failed to protect from bacteremia caused by BPF clone isolates. Thus, infant rats may be useful for assessing the virulence of *H. influenzae* biogroup aegyptius strains and for defining antigens that induce protective antibodies.

**CONCLUDING REMARKS**

On the basis of the work of Koch (20) and Weeks (40) in the late 19th century through the late 20th century, the pathogenic spectrum of *H. influenzae* biogroup aegyptius was thought to be limited to localized infections, usually involving the conjunctiva. The results presented here, however, show that *H. influenzae* biogroup aegyptius isolates of the BPF clone in Brazil differ from other *H. influenzae* biogroup aegyptius strains in several phenotypic properties and may therefore represent a new strain with unique pathogenic properties.

The studies reviewed here document the histopathological findings and attempt to correlate specific phenotypic characteristics with BPF. It has been shown that a single *H. influenzae* biogroup aegyptius clone is responsible for all of the cases of BPF in Brazil (7). The fact that a specific clone is associated with BPF and the enhanced virulence of BPF case isolates in infant rats compared with non-BPF clone isolates supports the argument that BPF clone isolates have specific virulence determinants not previously recognized in *H. influenzae* biogroup aegyptius strains. In addition, the histopathological and laboratory findings suggest that *H. influenzae* biogroup aegyptius endotoxin plays an important role in the toxic events that cause severe illness and death in patients with BPF.

Although certain protein and LOS phenotypes are present in BPF clone isolates, none has been shown to have a specific role in bacterial virulence. Thus, whether one (or more) of the identified phenotypic markers is associated with
virulence or is simply an epidemiological marker of a particular *H. influenzae* biogroup aegyptius strain requires further study. The BPF clone has been isolated from the conjunctivae, oropharynges, petechiae, blood, and hemorrhagic cerebrospinal fluid of patients with BPF (5–7, 18). It should be noted that not every patient with conjunctivitis due to *H. influenzae* biogroup aegyptius by the BPF clone develops BPF. Therefore, manifestation of BPF must depend on a series of events (entry, colonization, invasion, etc.) that are inextricably linked not only with the expressed virulence factors of *H. influenzae* biogroup aegyptius but also with host defenses. In this regard, except for a high association with preceding conjunctivitis, bacteremia, and subsequent pathological findings suggesting endotoxemia, the exact mechanism(s) of this age-dependent invasive disease in previously healthy children remains unknown.

Many fundamental questions have yet to be addressed with respect to the pathogenesis of BPF, including the following. How do *H. influenzae* biogroup aegyptius isolates of the BPF clone evade host defense mechanisms? What role does IgA1 protease play in this process? Where do the bacteria localize in a host after conjunctivitis and before disease? What roles do extracellular proteins, pili, and other membrane-associated proteins, LOS, and capsular polysaccharides play in the pathogenesis of BPF? The hope is that continued research into these and other areas will lead to improved strategies for the detection, prevention, and treatment of BPF.

ACKNOWLEDGMENT

We thank Eric Hansen for MAbs to *H. influenzae* LOS.

LITERATURE CITED


