Identification of *Haemophilus influenzae* Type b by a Monoclonal Antibody Coagglutination Assay

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A coagglutination assay using monoclonal antibody is described for the identification of *Haemophilus influenzae* type b. An immunoglobulin G2a monoclonal antibody, Hb-2, directed against a serotype-specific outer membrane protein of *H. influenzae* type b was adsorbed to *Staphylococcus aureus* Cowan 1 cells. In a dot enzyme immunoassay, Hb-2 reacted with 453 of 455 *H. influenzae* type b isolates and did not react with *H. influenzae* of other serotypes, untypeable *H. influenzae* strains, or other bacterial species. The Hb-2 coagglutination assay was evaluated by testing 136 *H. influenzae* type b strains selected on the basis of multilocus enzyme genotypes, 5 strains of another serotype, and 94 untypeable *H. influenzae* strains. The specificity of the coagglutination assay was demonstrated by the inhibition of the reaction by free Hb-2 monoclonal antibodies. The coagglutination assay was as specific as the dot enzyme immunoassay and can be rapidly performed and easily interpreted.

Haemophilus influenzae are gram-negative bacteria commonly found in healthy individuals, and six serotypes can be identified on the basis of capsular antigens (20). Serotype b is the most important pathogen, causing meningitis in young children, and some untypeable *H. influenzae* strains are responsible for certain serious infections in adults and children (16, 25, 27, 28). For identification of the etiologic agents implicated in these infections, serotyping by counterimmunoelectrophoresis, Quellung reaction, slide agglutination, and coagglutination are used (9, 19, 21, 28). These methods rely on the use of hyperimmune antisera containing antibodies directed against the type b capsular polysaccharide. Polyclonal antisera have lot-to-lot variability and cross-reactivity with several other bacterial species (3, 13, 23). Serotyping of untypeable strains as type b is an error usually encountered when slide agglutination, the most prevalent serotyping method, is used (24, 28). The use of coagglutination reagents for serological identification of clinical isolates of *H. influenzae* has proved more advantageous than conventional slide agglutination in many respects, including sensitivity and specificity (21, 24). Furthermore, when conventional antisera are used in serological assays, type b isolates can be misidentified when there is complete or partial loss of their capsular material after in vitro passages (5, 6, 15). As previously reported, the nutrient conditions influence the content of the capsule, and *H. influenzae* type b grown in defined medium may contain more capsular material than do bacteria grown in conventional broth (10). These difficulties can be overcome by using antibodies directed against a type-specific *Haemophilus* antigen other than the capsular antigen. This report describes a coagglutination assay which uses a monoclonal antibody directed against an accessible serotype-specific outer membrane protein for identifying *H. influenzae* type b.

Bacterial strains were grown on chocolate agar plates (I.A.F. Production Inc., Laval, Quebec, Canada) overnight at 37°C in a 5% CO₂ atmosphere. Most *H. influenzae* isolates were provided by pediatric research centers across Canada (7). The collection of *H. influenzae* type b strains also included 48 isolates from the United States, 52 from Europe, 9 from Africa, 10 from Papua New Guinea, 3 from Malaysia, 5 from the Dominican Republic, and 2 from Australia. These 129 isolates had been analyzed for multilocus enzyme variation as described by Musser et al. (18). *H. influenzae* strains were identified by their requirements for both X and V factors, and serotypes were determined by slide agglutination with commercially available sera (Difco Laboratories, Detroit, Mich.). Bacteria antigenically related to *H. influenzae* type b were also included (3, 13, 23).

The production and characterization of monoclonal antibodies directed against *H. influenzae* have been described previously (2, 7). Western immunoblotting analysis and ¹²⁵I-labeled protein A autoradiography revealed that monoclonal antibody Hb-2 (immunoglobulin G2a [IgG2a]) is directed against the 37,000-molecular-weight outer membrane protein (Fig. 1, lane C) and binds to protein A. In contrast, because Hb-1 is an IgG1, it does not bind to protein A (26) and hence cannot be detected by this method (Fig. 1, lane B). The results of competitive binding assay suggest that antibodies Hb-1 and Hb-2 are directed against the same or closely related epitopes.

The screening of monoclonal antibody Hb-2 against a large number of bacterial strains was performed by dot enzyme immunoassay as described by Hamel et al. (7). Briefly, cells from an overnight culture were suspended in phosphate-buffered saline (PBS), and 5 µl of the preparation was applied to nitrocellulose paper. The nitrocellulose paper was incubated with Hb-2, washed, incubated with peroxidase-conjugated anti-mouse immunoglobulins, and then developed in a solution of o-dianisidine.

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FIG. 1. Detection of antibody-specific epitopes on outer membrane proteins of *H. influenzae* type b. Outer membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7). After electrophoretic transfer, the blot was treated with 5% skim milk and then incubated with mouse hyperimmune sera (lane A), Hb-1 (lane B), or Hb-2 (lane C). Specific proteins were revealed by ^{125}I -labeled-protein A autoradiography. The numbers on the left are molecular weights in thousands.

The monoclonal coagglutination reagent was prepared by using commercial *Staphylococcus aureus* Cowan 1 cell suspensions (Pansorbin; Calbiochem-Behring Diagnostics, La Jolla, Calif.). A 0.1-ml volume of staphylococcal suspension was incubated with 0.2 mg of ammonium sulfate-precipitated Hb-2 for 2 min at room temperature, centrifuged, and then suspended in 0.5 ml of PBS. Control reagents were prepared in the same manner with monoclonal antibody Hb-1 or CMV-B1 instead of Hb-2. CMV-B1 (IgG2a) is specific to human cytomegalovirus (22). One or more colonies of the bacterial strain to be tested were suspended on a glass slide in one drop of PBS containing 100 mM glycine, 1 mM EDTA, and 0.5% gelatin. The coagglutination test was performed by adding 1 drop of the monoclonal antibody reagent to the bacterial suspension. The slide was rotated manually for 2 to 3 min, and the presence or absence of agglutination was recorded. The addition of gelatin in PBS eliminated nonspecific agglutination with non-type b isolates, and the EDTA improved the accessibility of the antigen at the cell surface, resulting in a faster and stronger agglutination reaction.

Because there are differences among *H. influenzae* type b isolates, as demonstrated by biotyping, outer membrane and lipopolysaccharide profiles, and multilocus enzyme genotypes (1, 11, 12, 14, 17, 18), the monoclonal antibodies were tested against genetically distinct type b isolates collected from several countries around the world. In the dot enzyme immunoassay, Hb-2 reacted with 453 of 455 *H. influenzae* type b strains. The nonreactive *H. influenzae* type b strains, one from Alaska and one from Norway, were of the uncommon electrophoretic types 30 and 29, respectively (18). Hb-2 did not react with encapsulated non-type b (15 isolates) and untypeable (131 isolates) *H. influenzae*, *Haemophilus parainfluenzae* (6 isolates), *Neisseria meningitidis* (11 isolates), group B streptococci (17 isolates), *Streptococcus pneumoniae* (12 isolates), *Escherichia coli* (12 isolates),

Staphylococcus epidermidis (9 isolates), or protein-A-negative *S. aureus* (8 isolates). The dot enzyme immunoassay results strongly suggest that Hb-2 is specific for *H. influenzae* type b. Hb-2 is also surface accessible (7). These are essential characteristics of a specific coagglutination reagent.

Hb-2 coagglutination reagent was used in the serotyping of 235 *H. influenzae* strains, including the 129 strains isolated from areas around the world and 106 strains from across Canada. All type b strains tested in the coagglutination assay gave a positive reaction. In reagent controls, no agglutination was observed with staphylococci sensitized with Hb-1 or CMV-B1. The Hb-2 coagglutination reagent showed no reaction with other capsular serotypes or untypeable *H. influenzae* or with *E. coli*, *S. epidermidis*, *N. meningitidis*, or *S. pneumoniae*.

The specificity of the coagglutination reaction with *H. influenzae* type b was determined by a competitive assay. When the *H. influenzae* type b suspensions were prepared in Hb-2 or Hb-1 ascitic fluids, the antigens reacted with free antibodies and no agglutination occurred when the coagglutination reagent was added. In contrast, there was agglutination when *H. influenzae* type b was suspended in HI-11 or CMV-B1 ascitic fluids. Monoclonal antibody HI-11 (IgG3) reacts with an epitope distinct from Hb-2 on *H. influenzae*. Agglutination with two untypeable strains was shown to be nonspecific, as determined by the agglutination reaction in the competitive coagglutination assay with free Hb-1 or Hb-2. These two strains did not agglutinate with the polyclonal antiserum in the slide agglutination test and did not react in the dot enzyme immunoassay with monoclonal antibody Hb-2. Uninterpretable results were obtained with two untypeable *H. influenzae* strains due to autoagglutination.

A rapid monoclonal antibody assay for identification of *H. influenzae* type b was developed. Monoclonal antibody Hb-2 identified *H. influenzae* type b by reacting specifically with a cell surface-exposed antigenic determinant common to more than 99% of the type b strains tested. The coagglutination assay, previously recommended by several investigators (8, 21, 24), was selected as a serotyping method on the basis of its known simplicity, accuracy, and rapidity. The affinity between Hb-2 and protein A makes this monoclonal antibody ideal as a coagglutination reagent. The monoclonal antibody Hb-2 coagglutination method does not depend on the presence of the capsule or intact organisms. Fresh isolates are not required in Hb-2 coagglutination tests because the outer membrane protein which reacts with Hb-2 is a stable component of the bacterial surface. Hb-2 detected *H. influenzae* type b antigens in isolates stored at 4°C or at room temperature and in outer membrane preparations kept frozen for several months. *H. influenzae* type b passed 17 times in culture or 24 times in mice (4) still reacted with Hb-2 in the dot enzyme immunoassay and coagglutination assay. The sensitivity of the coagglutination assay was demonstrated by the detection of 50 ng of protein, as determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada) using outer membrane from *H. influenzae* 3068 prepared as described previously (7). This rapid coagglutination assay for identification of *H. influenzae* type b is simple to perform, highly sensitive, and specific.

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LITERATURE CITED

1. Barenkamp, S. J., R. S. Munson, Jr., and D. M. Granoff. 1981. Subtyping isolates of *Haemophilus influenzae* type b by outer-membrane protein profiles. *J. Infect. Dis.* **143**:668-676.
2. Belmaaza, A., J. Hamel, S. Mousseau, S. Montplaisir, and B. R. Brodeur. 1986. Rapid diagnosis of severe *Haemophilus influenzae* serotype b infections by monoclonal antibody enzyme immunoassay for outer membrane proteins. *J. Clin. Microbiol.* **24**:440-445.
3. Bradshaw, M. W., R. Schneerson, J. C. Parke, Jr., and J. B. Robbins. 1971. Bacterial antigens cross-reactive with the capsular polysaccharide of *Haemophilus influenzae* type b. *Lancet* **i**:1095-1096.
4. Brodeur, B. R., P. S. Tsang, J. Hamel, Y. Larose, and S. Montplaisir. 1986. Mouse models of infection for *Neisseria meningitidis* B, 2b and *Haemophilus influenzae* type b diseases. *Can. J. Microbiol.* **32**:33-37.
5. Buckmire, F. L. A. 1976. Identification and quantitation of capsular antigen in capsulated and noncapsulated strains of *Haemophilus influenzae* type b by crossed-immunoelectrophoresis. *Infect. Immun.* **13**:1733-1742.
6. Escamilla, J. 1976. Susceptibility of *Haemophilus influenzae* to ampicillin as determined by use of a modified, one-minute beta-lactamase test. *Antimicrob. Agents Chemother.* **9**:196-198.
7. Hamel, J., B. R. Brodeur, Y. Larose, P. S. Tsang, A. Belmaaza, and S. Montplaisir. 1987. A monoclonal antibody directed against a serotype-specific, outer-membrane protein of *Haemophilus influenzae* type b. *J. Med. Microbiol.* **23**:163-167.
8. Himmelreich, C. A., S. J. Barenkamp, and G. A. Storch. 1985. Comparison of methods for serotyping isolates of *Haemophilus influenzae*. *J. Clin. Microbiol.* **21**:158-160.
9. Ingram, D. L., A. M. Collier, E. Pendergrass, and S. H. King. 1979. Methods for serotyping nasopharyngeal isolates of *Haemophilus influenzae*: slide agglutination, Quellung reaction, counterimmunoelectrophoresis, latex agglutination, and antiserum agar. *J. Clin. Microbiol.* **9**:570-574.
10. Inzana, T. J. 1986. A chemically defined medium induces resistance to lipopolysaccharide antibody in *Haemophilus influenzae* type b. *Microb. Pathogen.* **1**:483-489.
11. Inzana, T. J., and M. E. Pichichero. 1984. Lipopolysaccharide subtypes of *Haemophilus influenzae* type b from an outbreak of invasive disease. *J. Clin. Microbiol.* **20**:145-150.
12. Kilian, M. 1974. A rapid method for the differentiation of *Haemophilus influenzae* type b strains: the porphyrin test. *Acta Pathol. Microbiol. Scand. Sect. B* **82**:935-942.
13. Lagergård, T., and P. Branefors. 1983. Nature of cross-reactivity between *Haemophilus influenzae* types A and B and *Streptococcus pneumoniae* types 6A and 6B. *Acta Pathol. Microbiol. Immunol. Scand. Sect. C* **91**:371-376.
14. Loeb, M. R., and D. H. Smith. 1980. Outer membrane protein composition in disease isolates of *Haemophilus influenzae*: pathogenic and epidemiological implications. *Infect. Immun.* **30**:709-717.
15. Morse, S. I. 1980. The *Haemophilus-Bordetella* group, p. 694-700. In B. D. Davis, R. Dulbecco, H. N. Eisen, and H. S. Ginsberg (ed.), *Microbiology*, 3rd ed. Harper & Row, Publishers, Inc., New York.
16. Murphy, T. F., and M. A. Apicella. 1987. Nontypeable *Haemophilus influenzae*: a review of clinical aspects, surface antigens and the human immune response to infection. *Rev. Infect. Dis.* **9**:1-15.
17. Musser, J. M., S. J. Barenkamp, D. M. Granoff, and R. K. Selander. 1986. Genetic relationships of serologically nontypeable and serotype b strains of *Haemophilus influenzae*. *Infect. Immun.* **52**:183-191.
18. Musser, J. M., D. M. Granoff, P. E. Pattison, and R. K. Selander. 1985. A population genetic framework for the study of invasive diseases caused by serotype b strains of *Haemophilus influenzae*. *Proc. Natl. Acad. Sci. USA* **82**:5078-5082.
19. Myhre, E. B. 1974. Typing of *Haemophilus influenzae* by counterimmunoelectrophoresis. *Acta Pathol. Microbiol. Scand. Sect. B* **82**:164-166.
20. Pittman, M. 1931. Variation and type specificity in the bacterial species *H. influenzae*. *J. Exp. Med.* **53**:471-492.
21. Qadri, S. M. H., and D. Talbot. 1983. Evaluation of the Phadebact coagglutination test for the rapid serotyping of *Haemophilus influenzae*. *Med. Microbiol. Immunol.* **172**:161-164.
22. Rossier, E., K. Dimock, D. Taylor, Y. Larose, P. H. Phillips, and B. R. Brodeur. 1987. Sensitivity and specificity of enzyme immunofiltration and DNA hybridization for the detection of infected cells. *J. Virol. Methods* **15**:109-120.
23. Schneerson, R., M. Bradshaw, J. K. Whisnant, R. L. Myerowitz, J. C. Parke, Jr., and J. B. Robbins. 1972. An *Escherichia coli* antigen cross-reactive with the capsular polysaccharide of *Haemophilus influenzae* type b: occurrence among known serotypes, and immunochemical and biologic properties of *E. coli* antisera toward *H. influenzae* type b. *J. Immunol.* **108**:1551-1562.
24. Shively, R. G., J. T. Shigei, E. M. Peterson, and L. M. de la Maza. 1981. Typing of *Haemophilus influenzae* by coagglutination and conventional slide agglutination. *J. Clin. Microbiol.* **14**:706-708.
25. Trollfors, B., B. Claesson, T. Lagergård, and T. Sandberg. 1984. Incidence, predisposing factors and manifestations of invasive *Haemophilus influenzae* infections in adults. *Eur. J. Clin. Microbiol.* **3**:180-184.
26. Villemez, C. L., M. A. Russel, and P. L. Carlo. 1984. Mouse IgG1 heterogeneity: variable binding of monoclonal IgG1 antibodies to protein A-sepharose. *Mol. Immunol.* **21**:993-998.
27. Wallace, R. J., Jr., C. J. Baker, F. J. Quinones, D. G. Hollis, R. E. Weaver, and K. Wiss. 1983. Nontypeable *Haemophilus influenzae* (biotype 4) as a neonatal, maternal, and genital pathogen. *Rev. Infect. Dis.* **5**:123-135.
28. Wallace, R. J., Jr., D. M. Musher, E. J. Septimus, J. E. McGowan, Jr., F. J. Quinones, K. Wiss, P. H. Vance, and P. A. Trier. 1981. *Haemophilus influenzae* infections in adults: characterization of strains, serotypes, biotypes and β -lactamase production. *J. Infect. Dis.* **144**:101-106.