Significance of Noncapsular Antigens in Protection Against Experimental *Haemophilus influenzae* Type b Disease: Cross-Reactivity

RICHARD L. MYEROWITZ,[1,2]* CARL W. NORDEN,[2,3] AND THOMAS A. DEMCHAK[1,2]

Department of Pathology, Presbyterian-University Hospital,[1] Department of Medicine, Montefiore Hospital,[3] and the University of Pittsburgh School of Medicine,[2] Pittsburgh, Pennsylvania 15213

A bacterial strain, tentatively identified as an *Actinobacillus* species, was found to asymptomatically colonize the pharynx of some rats and to bear cell wall antigens which cross-react with noncapsular antigens of *Haemophilus influenzae* type b (HIb). The cross-reacting antigens appeared to be a heterogeneous mixture with varying molecular size and charge. The antigenic moieties are probably carbohydrate in nature. Antisera raised with this strain had both immunochemical and biological (bactericidal, opsonizing, and protective against experimental infection) activity against HIb. These findings lend further evidence to the idea that noncapsular antigens are important in the induction of resistance to HIb disease. The findings also raise the possibility of using bacteria which cross-react with noncapsular antigens for immunization against HIb disease in humans through nasopharyngeal or enteric colonization.

The pioneering study of Fothergill and Wright (3) showed that immunity to *Haemophilus influenzae* type b (HIb) was age-related and associated with bactericidal (BA) antibodies. These BA antibodies were assumed to be protective against disease and were subsequently shown to be directed primarily at the HIb capsular polysaccharide antigen (1, 23). However, several lines of evidence have suggested that anticapsular (AC) antibodies are not solely responsible for protective immunity to HIb. Not all of the BA activity from human or animal serum can be absorbed with purified capsular polysaccharide antigen (1). In many sera, BA antibody activity does not parallel AC antibody content (10, 15). By using passive protection experiments in infant rats, a small but reproducible amount of protective activity in hyperimmune burro anti-HIb serum was shown to be due to noncapsular antibodies (13). Finally, Granoff and Rockwell (4) have shown and we have confirmed (R. L. Myerowitz and C. W. Norden, Br. J. Exp. Pathol, in press) that the offspring of rat survivors of infantile HIb disease are protected from experimental HIb infection by BA antibodies directed against noncapsular antigens.

A major portion of recent HIb research has dealt with cross-reacting antigens. Schneerson et al. (21) and Robbins et al. (20) have described enteric bacteria with surface polysaccharide antigens which cross-react with the capsular polysaccharide of HIb and other encapsulated pyogenic bacteria. Gastrointestinal colonization by these bacteria has been suggested to be the antigenic source for the age-related acquisition of AC antibodies, which occurs in the absence of infection with HIb ("natural immunity"). Experimental gastrointestinal colonization of animals and humans with these bacteria has resulted in either an AC antibody response or sensitization of the host to make a greater-than-expected AC antibody response after subsequent HIb infection (5, 11, 14, 22). Since some of the "natural immunity" to HIb is due to noncapsular antibodies, it is conceivable that asymptomatic colonization with bacteria that cross-react with such noncapsular HIb antigens may be the antigenic stimulus for these antibodies.

Recently Smith et al. (24) and Moxon et al. (9) described an experimental model of HIb meningitis in infant rats using intraperitoneal and intranasal inoculations, respectively. In studies from our laboratory (6, 12), using the intranasal model, we were able to produce disease in rats, but not with as great frequency as Moxon. This apparent resistance to disease among rats used by us went unexplained until we noted that by the antiserum-agar technique (8) pharyngeal cultures from all rats obtained from our supplier were positive for an organism (Hilltop strain [HT]) that cross-reacts with noncapsular antigens of HIb. This report describes our studies investigating the bacteriological identity of HT, the specificity of the cross-reacting antigen and its chemical nature, and the

ability of this strain to induce antibodies which have biological activities (bactericidal, opsonic, and protective) against HIb.

MATERIALS AND METHODS

Experimental animals. Outbred pathogen-free, albino Sprague-Dawley rats were purchased from Hilltop Lab Animals, Scottsdale, Pa., and housed in a single room at the Children's Hospital of Pittsburgh.

Bacteriological methods. The techniques of culture and storage of HIb strain Pekala have been described previously (12). The unencapsulated strain of *H. influenzae* (HI), which was used in this study (strain F2), was isolated from a child with otitis media; HT was isolated from the pharynx of our rats. Both organisms were stored frozen at −70°C. The biochemical tests used to identify HT utilized standard media into which 10% horse serum has been incorporated. HIb strain Pekala, HI strain F2, and HT were incubated in candle jars when grown on solid medium. *Escherichia coli* 075:K100:H5 (strain Easter) was the same strain as that used in previous studies (21, 22). Sonic oscillation of bacteria was carried out on bacterial suspensions in phosphate-buffered saline (PBS; 0.05 M, pH 7.1) prepared from plates with confluent growth after overnight incubation (1 ml of PBS per 150-mm petri dish). Portions of 5 ml were sonically treated for 10 s in an ice bath using a Sonic-Dismembrator (Quigley-Rochester, Rochester, N.Y.) set at 40. The procedure was repeated six times for each portion. Sonic extracts were clarified at 14,000 × *g* in a Sorvall refrigerated centrifuge. Ultrastructural studies of overnight broth cultures were done by using negative staining with 2% phosphotungstic acid and a Phillips EM 200 electron microscope.

Biochemical methods. Diethylaminoethyl (DEAE)-cellulose chromatography was performed using DE 52 resin (Whatman, Kent, England) in a column with a bed volume of 25 ml and a starting buffer of 0.01 M phosphate (pH 7.0). The sample was applied after overnight dialysis in 500 volumes of buffer. The flow rate was 0.5 ml/min and fraction size was 6.6 ml. The gradient buffer (300 ml) consisted of 0.00 M–0.30 M NaCl in starting buffer. Fractions were analyzed for protein content by measuring their absorbancy at 280 nm in a Beckman DU spectrophotometer and for antigenic reactivity by counterimmunoelectrophoresis (CIE) with burro anti-HIb serum.

Quaternary aminoethyl (QAE)-cellulose chromatography was performed using A-50 resin (Pharmacia Fine Chemicals, Piscataway, N.J.) in a column with a 10-ml bed volume. The starting buffer, sample application, flow rate, and fraction size were identical to those used above. A 100-ml portion of an initial gradient (0.00 M–0.30 M NaCl in starting buffer) was applied followed by another 100 ml of a second gradient (0.30 M–0.50 M NaCl in starting buffer). Fractions were analyzed as outlined above.

Gel filtration was done with Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) in a column (3 by 20 cm) of approximately 50-ml bed volume. The flow rate was 2.5 ml/min and the fraction size was 2 ml. Fractions were analyzed as outlined above.

Trypsin digestion utilized 80 μl of antigen solution (1 mg/ml) mixed with 10 μl of 0.5 M phosphate buffer (pH 7.2) and 10 μl of trypsin solution (1 mg/ml, P-L Biochemicals, Milwaukee, Wisc.). The mixture was incubated for 2 h at 37°C. A 10-μl portion of alpha 1-antitrypsin solution (5 mg/ml) was added to stop the reaction. A blank solution containing all reagents except trypsin served as a control. Periodate oxidation was carried out with 20 μl of 0.5 M sodium metaperiodate added to 80 μl of antigen solution and incubated for 24 h at 4°C in the dark. A 10-μl portion of glycerol was added to stop the reaction. This mixture was incubated for an additional 8 h at 4°C.

Immunological methods. Immunodiffusion analysis was performed as described (20) by using crude saline extracts as antigens. The saline extracts consisted of a dense bacterial suspension prepared by emulsification of a single colony from an overnight agar plate culture into 0.1 ml of PBS and incubated at room temperature for 1 h. The antiserum-agar technique (8) and CIE (7) were carried out as described. End-point dilution titrations by CIE were done using a 10-mg/ml solution of HT or HI sonic extract. Immunoelectrophoresis was carried out as described (21), except that the tank and gel buffers were identical to those used for CIE. Passive protection assays in rats were similar to those previously described (13), except that only small litters were used (10 rats per litter) and the challenge inoculum was 10² colony-forming units (CFU). The use of smaller litters and a lower inoculum increased the sensitivity of the assay so that the reciprocal 50% end-point protective titer (19) of hyperimmune burro antiserum to HIb was raised from 1,750 to 7,000. Absorption of antisera with Formalin-killed whole bacteria utilized an overnight broth culture (10 ml of broth per ml of serum), which was sedimented by centrifugation and suspended in 2% phosphate-buffered Formalin. After overnight incubation at 4°C, the suspension was washed three times in PBS. Absorption with purified antigen used lyophilized material suspended directly in serum. All absorptions were carried out at 37°C for 2 h followed by incubation at 4°C for 24 h. Immunization of rabbits with Formalin-killed bacteria and immunization with purified antigen (50 μg) in Freund adjuvant (Difco) were carried out as described (13, 26). BA (16), AC (17), and opsonizing (4) antibodies to HIb were determined as described. Pooled normal rabbit serum was obtained from GIBCO (Grand Island, N.Y.).

RESULTS

Bacteriological identification of HT. HT is a short (0.4 to 1 μm), moderately pleomorphic, gram-negative, nonmotile, nonencapsulated (by electron microscopy) bacillus which is capnophilic and requires 10% serum for optimal growth. Its biochemical and growth characteristics are not similar to those of any medically important human enteric species (2). HT has weakly positive oxidase and catalase properties and positive nitrate reduction. Motility, indole, urease, deoxyribonuclease, hydrogen sulfide, esculin hydrolysis, and gelatinase are negative. X and V factors are not required. Of the sugars,

those that ferment slowly are dextrose, sucrose, maltose, xylose, and fructose. Those that do not ferment are lactose and mannitol. Rabbit blood, sheep blood, chocolate, Leventhal, and Trypticase soy agars support growth. Agars inhibiting growth include MacConkey, salmonella-shigella, Hektoen enteric, Columbia nalidixic, and Thayer-Martin. According to Bergey's Manual (18), these characteristics most closely fit an *Actinobacillus* species and differ in only a few reactions from that of *Actinobacillus equuli.*

Specificity and site of the cross-reacting antigen. Inoculation of HT on HIb antiserum-agar produced halos only after at least 3 days of incubation at room temperature. The sonic extract of HT produced a double precipitin band in immunodiffusion analysis with hyperimmune burro anti-HIb serum (Fig. 1). This band formed an identity reaction with the minor bands of an HIb saline extract. These minor bands were due to noncapsular antigens as shown by immunodiffusion analysis with purified HIb capsular antigen. At least four bands were seen in CIE analysis of the HT sonic extract with the same antiserum. A reaction of nonidentity was observed between the HT sonic extract and the surface K100 antigen-containing saline extract of *Escherichia coli* strain Easter. The K100 antigen partially cross-reacts with the HIb cap-

sular polysaccharide (21). No reaction was observed with HT sonic extract in immunodiffusion analysis with hyperimmune antisera to meningococcus groups A or C (horse and burro, respectively) or burro antiserum to *E. coli* strain Easter. Absorption of the burro anti-HIb serum with purified capsular polysaccharide failed to remove any reactivity with HT. Comparison of HT antigens with those of unencapsulated HI strain F2 (Fig. 2) revealed that the HT antigens are at least partially shared with the HI strain, but that the latter had other antigens not shared by HT. This observation was confirmed by absorption of burro anti-HIb serum with the HT sonic extract, which removed all reactivity with HT but left some precipitin activity to HI strain F2. Absorption with the HI strain removed reactivity to both HI and HT. Absorption of burro anti-HIb serum with Formalin-treated, whole HT failed to remove any reactivity with HT, whereas absorption with the HT sonic extract removed all reactivity. This latter result suggests that the cross-reacting antigen is situated deep within the cell wall of HT since sonic treatment was required to release sufficient antigen to be used as an absorbent.

Purification and chemical properties of the cross-reacting antigen. The cross-reacting antigens of HT were stable when heated at
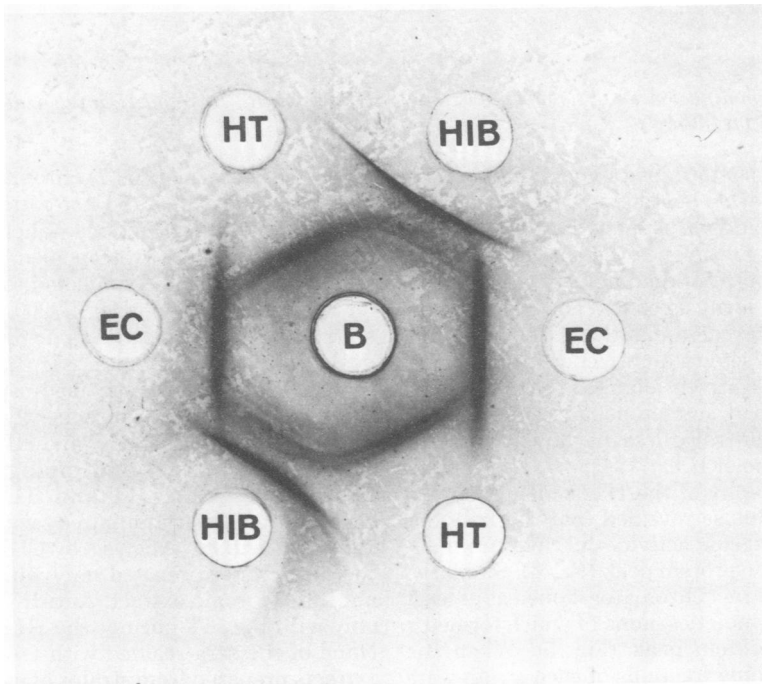

FIG. 1. *Immunodiffusion analysis using burro anti-HIb serum (B), HT sonic extract (10 mg/ml), and saline extracts of H. influenzae b strain Pekala (HIb) and E. coli strain Easter (EC).*
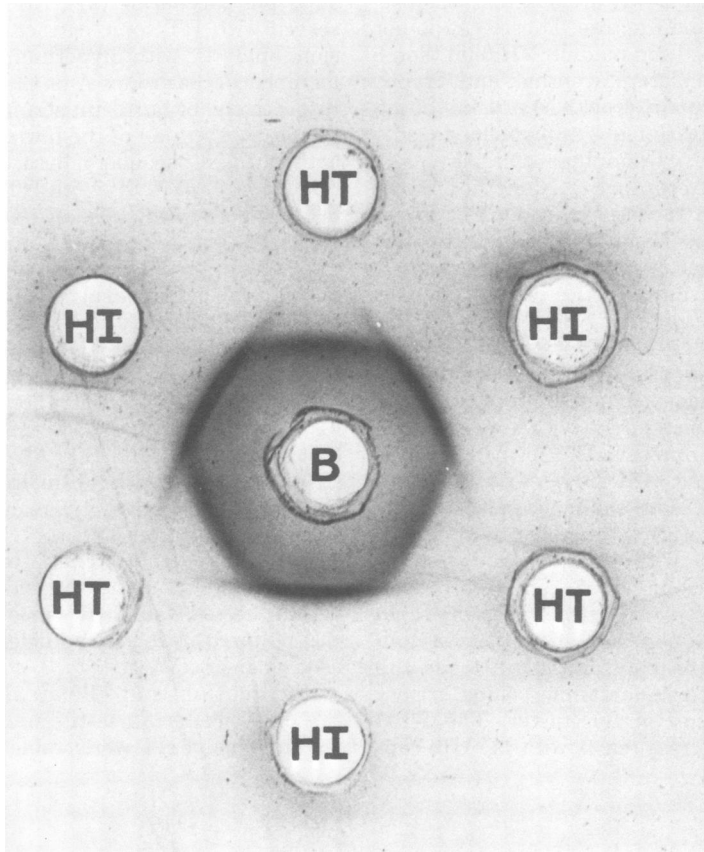
FIG. 2. *Immunodiffusion analysis using burro anti-HIb serum (B), HT, and H. influenzae strain F2 (HI) sonic extract (10 mg/ml).*

37°C for 1 h as determined by immunodiffusion analysis. However, heating at 56°C for 30 min destroyed the antigen's reactivity. Periodate oxidation likewise destroyed the antigen's reactivity, but trypsinization had no effect. Immunoelectrophoretic analysis of HT and HI sonic extracts (Fig. 3) revealed that the HT sonic extract contained five separate antigenic species, all of which were negatively charged. The HI sonic extract also had numerous antigenic species which were generally less negatively charged than were those of HT.

Chromatography of the HT sonic extract using DEAE-cellulose revealed that the fractions containing antigenic activity did not chromatograph as a single protein peak (Fig. 4). Similarly, the HI sonic extract chromatographed as at least three separate moieties, none of which formed a recognizable protein peak (Fig. 5). When the antigen-containing fractions of each strain were pooled and subjected to further chromatography with QAE-cellulose, both the HT antigens (Fig.

6) and HI antigens (Fig. 7) chromatographed as a broad smear which did not correspond to the protein-containing fractions. When the antigen-containing fractions were pooled, concentrated by lyophilization, and subjected to gel filtration on a column of Sephadex G-200, the antigens eluted as a broad smear with no definite protein peak. The antigen-containing fractions from both HT and HI were pooled separately, and these materials were subsequently used for immunization as purified HT and HI antigens.

Immunochemical and biological properties of antisera to HT and HI. Two rabbits each were hyperimmunized with Formalin-killed HT and HI. Analysis by CIE showed that the anti-HI sera reacted only with the HI but not the HT sonic extract. Anti-HT sera reacted only with the HT but not the HI sonic extract. None of the sera reacted with any of the sonic extracts prepared from strains of meningococcus groups A, B, and C, *Shigella flexneri*, *E. coli* strain Easter (075:K100:H5), and *E. coli* 07:K1.
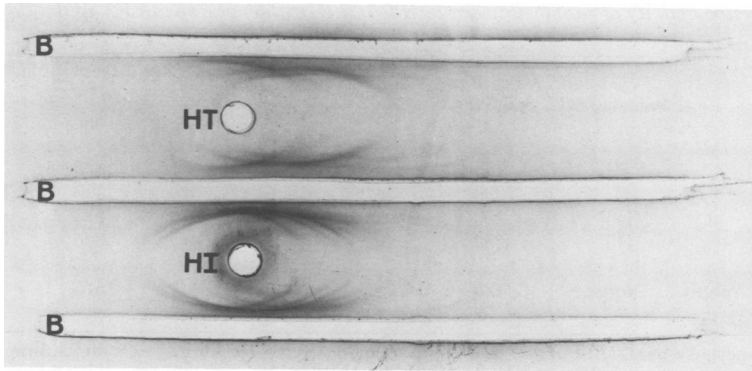
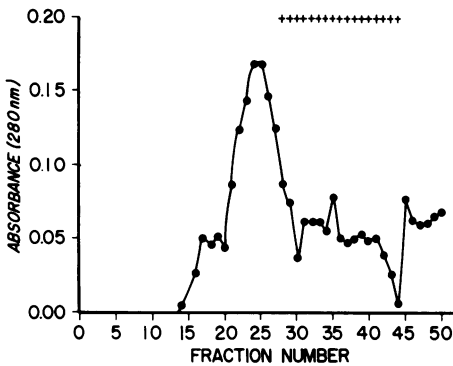FIG. 3. *Immunoelectrophoresis of HT and H. influenzae strain F2 (HI) using burro anti-HIb serum (B).*

FIG. 4. *Chromatogram of HT sonic extract on DEAE-cellulose. +++, Fractions with antigenic activity.*
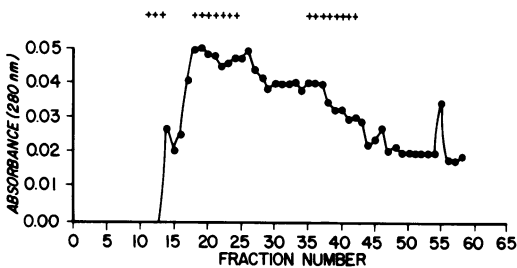

FIG. 5. *Chromatogram of H. influenzae strain F2 sonic extract on DEAE-cellulose. +++, Fractions with antigenic activity.*
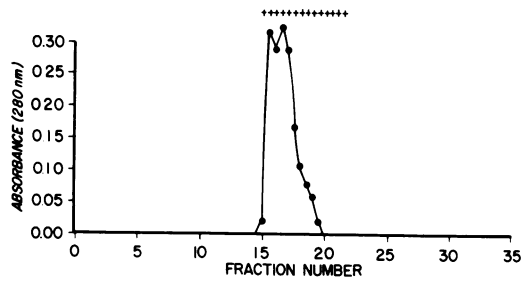

FIG. 6. *Chromatogram of HT antigens (from DEAE-cellulose chromatography) on QAE-cellulose. +++, Antigenic activity.*
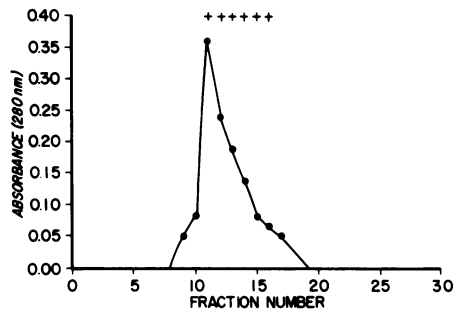

FIG. 7. *Chromatogram of H. influenzae strain F2 antigens (from DEAE-cellulose chromatography) on QAE-cellulose. +++, Antigenic activity.*

All hyperimmune sera had some BA activity toward HIb strain Pekala. A small but reproducible protective effect was also observed with all sera (Table 1). Unfortunately, pooled normal rabbit serum had to be used as a control since preimmunization serum samples were not collected from the immunized rabbits. This control serum had no protective activity despite a concentration of AC antibodies and BA activity toward HIb strain Pekala that was similar to the hyperimmune sera. One hyperimmune serum

was tested and had opsonizing activity toward HIb which was elevated above the control serum (Table 2). Absorption of one of the anti-HT sera with the HT sonic extract, sufficient to remove precipitin reactivity, also absorbed all protective activity from that serum. No protective effect was observed with hyperimmune rabbit antiserum to *S. flexneri* (agglutination titer, 1:10,000) or Difco antiserum to *E. coli* O125:K70.

Three of the four rabbits were allowed to rest for 2 months after bacterial immunization and were then immunized with 50 μg of either puri-

TABLE 1. *Protective activity of rabbit antisera to HT and H. influenzae strain F2 against experimental HIb disease in infant rats*

Rabbit no.	Immunogen	AC antibody concn (ng/ml)	BA[a] antibody	Protective effect of serum dilution				
				Undiluted	1:2	1:4	1:8	1:16
1	HI	155	±	1/5[b]	1/5	2/5	5/5	ND[c]
2	HI	255	±	0/5	0/5	1/5	5/5	ND
3	HT	100	±	0/5	0/5	0/5	4/5	5/5
4	HT	355	±	0/5	0/5	2/5	5/5	5/5
Pooled normal rabbit serum	None	120	±	5/5	5/5	5/5	5/5	ND

[a] Bactericidal activity to strain Pekala. +, >75% killing; ±, 40 to 75% killing; −, <40% killing.
[b] Number bacteremic per total number tested.
[c] ND, Not done.

TABLE 2. *Opsonizing activity against HIb of rabbit anti-HT serum*

Rabbit antiserum	AC antibody concn (ng/ml)	% Opsonization[a]
Anti-HT serum (Rabbit 3)	100	119.7
Pooled normal rabbit serum	120	73.6
Anti-HIb	1.4×10^6	493
Pooled normal rat serum	None detected	13.5

[a] Results are calculated by the following formula: $(X - M/CP - M) \times 100$. X = Mean of triplicate cpm obtained with unknown sample; M = mean of triplicate cpm obtained with media control; CP = mean of triplicate cpm obtained with 100% control (serum from rats who have survived infantile HIb disease).

fied HT or HI antigen in Freund adjuvant. Analysis of the sera from before and after this second immunization for precipitating antibodies (using CIE with end-point dilution titration) and protective activity (Table 3) revealed that none of the rabbits had responded to this form of immunization.

DISCUSSION

This report has described a bacterial strain, tentatively identified as an *Actinobacillus* species, which colonizes the pharynx of some rats and which cross-reacts with noncapsular antigens of HIb. This strain is capable of inducing antibodies which have both immunochemical and biological activities against HIb. These data add further evidence to the hypothesis that noncapsular antigens of HIb are significant in inducing resistance to HIb disease.

The passive protection experiments performed in this study were carried out with infant rats derived from the same supplier as all other rats in these experiments. These rats have mothers that are colonized with HT and which probably have background low levels of resistance to HIb disease, which is passed to their offspring

via colostrum. The lack of protective activity in the control normal rabbit serum is, therefore, an especially important observation because it indicates that the protective activity of hyperimmune anti-HT sera was not due to this background resistance. The finding of low levels of BA (Table 1) and opsonizing (Table 2) activities in this control serum is not surprising since most adult rabbits have antibodies to HIb (11). The absence of protective activity in this serum may be due to a lower sensitivity of the protection assay as compared with the BA and opsonizing assays. Also, it may not always be possible to predict with certainty from in vitro phenomena, such as the BA reaction and opsonization, whether such antibody will be protective in vivo.

These observations also raise the following important questions. (i) What is the role of such cross-reacting strains in the age-related acquisition of BA antibodies to HIb in humans? Surveys of human flora, e.g., from pharynx or stool, should be carried out by the antiserum-agar technique to determine the prevalence of bacteria which cross-react with noncapsular HIb antigens. (ii) What is the role of such strains in producing resistance to experimental HIb disease? Whether colonization with HT is responsible for the lower frequency of intranasally induced HIb disease in our rats compared with those of Moxon is still an open question. Preliminary investigation has shown that infant and adult rats purchased from the supplier used by Moxon (Charles River Breeding Laboratories, Wilmington, Mass.) are not colonized with HT. Simultaneous inoculation with HIb of rats from both suppliers should answer this question. (iii) Can deliberate colonization by HT or other cross-reacting strains induce resistance to HIb disease? If feeding experiments in rats are successful, then the possibility of using these strains as immunogens in humans will be raised.

The nature of the cross-reacting antigens requires further study. The present results suggest

TABLE 3. *Precipitating antibodies and protective activity of antisera to HT and HI after immunization with purified antigen in Freund adjuvant*

Rabbit no.	Immunogen	AC antibody concn (ng/ml)	CIE titer	Protective effect serum dilution			
				1:2	1:4	1:8	1:16
2 (Preimmune)[a]		255	1/4	0/5[b]	1/5	5/5	ND[c]
2 (Postimmune)	HI	330	1/8	0/5	0/5	3/5	5/5
3 (Preimmune)[a]		100	1/8	0/5	0/5	4/5	5/5
3 (Postimmune)	HT	380	1/8	0/5	2/4	4/5	5/5
4 (Preimmune)[a]		355	1/16	0/5	2/5	5/5	5/5
4 (Postimmune)	HT	250	1/4	0/5	1/5	3/5	5/5

[a] These rabbits had been previously immunized with whole killed HT but were preimmune with respect to immunization with purified antigen in Freund adjuvant.

[b] Number bacteremic per total number tested.

[c] ND, Not done.

that these antigens are a heterogeneous mixture of molecules with different molecular sizes and charges. The ability of periodate but not trypsin to destroy antigenic activity suggests that the antigenic moieties are carbohydrate in nature. The inability to stimulate antibody production in rabbits by immunization with purified antigen in Freund adjuvant is also consistent with the nonprotein nature of the antigens. If these antigens are polysaccharides, then their potential usefulness as vaccines, at least in purified form, would probably be limited since other polysaccharide vaccines have proven to be poorly immunogenic in infants (25). The possibility that one or all of these cross-reacting antigens are shared by all gram-negative bacteria (e.g., endotoxin) appears unlikely since other gram-negative strains do not produce halos on antiserum agar, HT does not produce precipitin bands with anti-meningococcus antisera, and antisera to *E. coli* O125:K70 and *S. flexneri* had no protective activity against HIb disease.

ACKNOWLEDGMENTS

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