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Cross-Reactivity of Surface-Exposed Epitopes of Outer Membrane Antigens of *Haemophilus influenzae* Type b

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The cross-reactivity of exposed surface epitopes of outer membrane proteins from a spectrum of *Haemophilus influenzae* type b isolates that varied in their evolutionary distance from each other and in their outer membrane protein composition was analyzed by using an immunoblot assay. The results for outer membrane proteins *a*, *n*, and *b/c* were as follows. (i) A total of 13 of 14 strains possessing a protein *a* with similar mobilities on gels (i.e., the same apparent molecular weight) as protein *a* of strain Eag absorbed antibodies to protein *a* of strain Eag. These strains represented a broad spectrum on a scale of evolutionary distance. (ii) In contrast, only one of seven strains possessing a protein *a* with different mobilities absorbed these antibodies. (iii) Of five isolates close to strain Eag on the evolutionary scale, the four with a protein *n* with the same mobility as protein *n* of strain Eag absorbed antibodies to protein *n* of strain Eag. (iv) In contrast, of five isolates distant from strain Eag on the evolutionary scale, none absorbed antibodies to protein *n*, including one strain that had a protein *n* of the same mobility as that of strain Eag. (v) All strains that absorbed antibodies to protein *b/c* also absorbed antibodies to lipopolysaccharide, and the reverse of this was also true. Evolutionary distance and mobility of protein *b/c* on gels were not factors. Control experiments indicated that this result was an artifact due to the strong association of lipopolysaccharide with protein *b/c* on the gel and subsequent blot. The important conclusions from these experiments, especially pertinent for consideration of these proteins in either whole or peptide vaccines, are that proteins with apparently identical molecular weights can possess different surface-exposed epitopes, that proteins with different molecular weights can possess cross-reactive surface-exposed epitopes, and that some surface-exposed epitopes have been conserved even though the bacterium has undergone evolutionary divergence. In addition, experiments were also performed to determine whether *H. influenzae* type b strains maintained their integrity during the absorption step, i.e., incubation in antiserum. Strain Eag, which was used as a prototype type b strain, released a small proportion of its membrane (0.13%), but this did not result in exposure of epitopes that were usually buried. In contrast, strain S2, an unencapsulated mutant of strain Eag, was quite unstable, releasing three times as much membrane and a large proportion of its periplasmic proteins.

Haemophilus influenzae type b is a gram-negative, encapsulated bacterium which causes serious systemic disease, primarily in young children. Because a recently licensed vaccine consisting of the capsular polysaccharide does not induce protective levels of antibodies in children under 24 months of age (10), an alternate and more effective vaccine is needed. One group of plausible vaccine candidates are the outer membrane proteins (OMPs) of *H. influenzae* type b, since individuals of all ages can make antibodies to these cell surface antigens (5, 15) and since antibodies to some of these proteins are bactericidal (13a), protective, or both, as demonstrated with the infant rat model of *H. influenzae* disease (for a review, see reference 3; 13a).

The outer membrane of *H. influenzae* type b is typical of a gram-negative bacterium; it contains about two dozen proteins, with six, the major OMPs accounting for about 80% of the total OMPs (17). In our laboratory these proteins are referred to as *a* (46,000-molecular-weight-protein [46K protein]; *b/c*, a doublet (37K protein); *d/f* (two conformers of the same protein whose molecular weights are 33,000 and 26,000); *e* (28K protein); and *g* (16K protein). An alternate nomenclature is, respectively, P1, P2, P5, P4, and P6 (3). The molecular weights noted above are based on mobility on sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis (PAGE) of outer membranes derived from our standard laboratory strain of *H. influenzae* type b, strain Eag (17). Although, as determined by mobility on SDS-PAGE, proteins *a*, *b/c*, and *d/f* vary among *H. influenzae* type b strains and proteins *e* and *g* appear to be the same in all strains (14), essentially nothing more is known of their comparative biochemistry. In considering an OMP in a vaccine, one critical factor that is more significant than molecular weight similarities is the extent of cross-reactivity of exposed (i.e., surface) epitopes. The ideal candidate would have identical surface epitopes among all strains, and such epitopes would induce protective antibodies. Very limited data regarding cross-reactivity are available, and they have been obtained for only a very few type b strains in infant rat protection studies, which are expensive and too time-consuming for extended studies on cross-reactivity (12, 19).

A preferred assay would enable comparison of the cross-reactivity of several outer membrane antigens from an extensive group of isolates. Consequently, in our laboratory we have performed experiments in which we evaluated existing methods and developed an alternate method, the immunoblot method, for accomplishing this (13, 16). In this report we describe data obtained by the immunoblot assay on the cross-reactivity of some outer membrane components of strain Eag with those from a spectrum of *H. influenzae* type b strains. Strains were selected for analysis from the 24 established OMP subtypes based on distinct mobility profiles in gels due to proteins *a*, *b/c*, and *d/f* (1, 20) and from the 32 electrophoretic types (ETs) based on distinct mobility differences of 16 metabolic enzymes on starch gel electrophoresis

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(20), with a spectrum of evolutionary distance extending from ET1 to ET32.

MATERIALS AND METHODS

Bacterial strains and growth. Strains Eag (type b), S2 (an unencapsulated mutant of strain Eag), and C353 (untypable) were from the University of Rochester collection. All other strains were type b and are listed in Table 1. They were obtained from the collection of D. M. Granoff (Washington University, St. Louis, Mo.) and were obtained with his permission from J. Musser (University of Rochester). They represented isolates from various anatomic sites and all were from the United States, except for strain 1064, which was from Thailand. Information on OMP subtypes and ETs were kindly provided by D. M. Granoff and J. Musser. Strains were stored as skim milk stocks at −80°C and grown to 5×10^8 CFU/ml in brain heart infusion medium (Difco Laboratories, Detroit, Mich.) supplemented with 1 to 10 μg of hemin per ml and 2 μg of NAD (Sigma Chemical Co., St. Louis, Mo.) per ml as described previously (17).

Antisera. Two preparations of rabbit antisera, one (R99) to live strain Eag and the other (R102) to the S2 mutant of Eag, were obtained by multiple intravenous immunizations as described previously (13); one of these was generously donated by P. Anderson.

TABLE 1. ETs and OMP subtypes of strains of *H. influenzae* type b used for immunoblot assay of cross-reactivity of exposed surface epitopes

Row	Strain	ET[a]	OMP subtype[b]
1	1080	4	1L
2	1085 (Eag)	6	1L
3	1075	8	1L
4	1052	10	9L
5	1079	11	2L
6	1060	12	3L
7	1064	12	1L
8	1072	13	3L
9	1071	19	3L
10	1078	20	3L
11	1053	21	13L
12	1068	22	11L
13	1062	23	11L
14	1059	24	5L
15	1063	27	6U
16	1054	1	2H
17	1061	7	1H
18	1073	9	1H
19	1065	25	6U
20	1069	30	8H
21	1084	32	17H
22	811	1	7H
23	1101	1	10H
24	1034	1	15L
25	1132	1	4H
26	1391	1	19L
27	1251	12	18L
28	1058	15	14L
29	1055	17	16L
30	649	21	11L
31	1148	30	8H

[a] ETs are based on polymorphisms of 16 enzymes, as determined by starch gel electrophoresis (20).

[b] OMP subtypes were determined by D. M. Granoff and co-workers (1, 20) and are based on mobility differences of proteins P1 (*a*), P2 (*b*/*c*), P4 (*e*), and P5 (*d*/*f*) (our nomenclature is given in parentheses) on SDS-PAGE. The H, L, and U components of the OMP subtypes refer to the mobility of protein P1 (*a*). Most type b strains fall into the H or L subtypes; a few (U) are outside this classification.

Absorption of antisera. Absorption of antisera has been described previously in detail (13). Briefly, 0.8 ml of heat-inactivated rabbit antisera at a 1:100 dilution in 0.01 M sodium phosphate–0.15 M NaCl (pH 7.2)–0.5 mM $MgCl_2$–0.15 mM $CaCl_2$ (PCM) and 3.5×10^{10} CFU of *H. influenzae* type b grown to 5×10^8 CFU/ml in supplemented brain heart infusion medium were mixed, kept on ice for 30 min, and then centrifuged to obtain the absorbed antiserum. In some instances, to remove a high background due to anti-lipopolysaccharide (anti-LPS) antibodies, the serum was first depleted of anti-LPS by affinity chromatography on a column of Sepharose-4B containing covalently bound LPS (22).

Immunoblot assay for cross-reactivity of exposed determinants. The method for the immunoblot assay for cross-reactivity of exposed determinants has been described previously in detail (13). Briefly, an outer membrane preparation from strain Eag (17) was subjected to SDS-PAGE, and the individual proteins and LPS, as separated on the gel, were then electroblotted onto nitrocellulose. The nitrocellulose was cut into vertical strips; one strip was incubated with unabsorbed antiserum to strain Eag, while another was incubated with the same antiserum after it was absorbed (as described above) with a different strain of *H. influenzae* type b. After incubation of the strips with ^{125}I-labeled staphylococcal protein A and subsequent autoradiography, the intensity of the antibody reaction to individual components on strips exposed to unabsorbed and absorbed antisera was compared. A decrease in intensity of a particular band demonstrated that the different strain absorbed antibodies to that component, thereby indicating the cross-reactivity of the surface-exposed part(s) of that antigen between that strain and strain Eag.

Stability of bacteria. To determine the stability of selected *H. influenzae* strains in antiserum, the bacteria were grown to 5×10^8 CFU/ml in supplemented brain heart infusion medium containing 8.3 to 12.5 μCi of L-[4,5-^{3}H]leucine (63 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml. Unless stated otherwise the bacteria were chilled, divided into 35-ml fractions, and collected by centrifugation for 10 min at 10,000 × *g*. Each pellet was suspended in 16 ml of PCM, collected by centrifugation, suspended in 1 ml of PCM, transferred to a microcentrifuge tube (1.5-ml volume), centrifuged at 8,000 × *g* for 5 min, and then suspended in 0.4 ml of PCM or antiserum. After absorption was carried out as described above (30 min, 0°C), the suspension was centrifuged at 8,000 × *g* for 15 min, and the supernatant was collected and centrifuged again. The final supernatant was collected and assayed for released radioactivity as follows. (i) The amount of radioactivity released during absorption was determined by adding 0.1 ml of the PCM or absorbed antisera supernatant to 10 ml of Scintiverse II (Fisher Scientific Co., Pittsburgh, Pa.) and by counting this mix in a scintillation counter (model 3375; Packard Instrument Co., Inc., Rockville, Md.). (ii) The released radioactivity was size fractionated into high- and low-molecular-weight material on a 2-ml column of Bio-Gel P2 (Bio-Rad Laboratories, Richmond, Calif.) in a buffer of 0.01 M sodium phosphate–0.15 M NaCl (pH 7.2; phosphate-buffered saline)–0.1% SDS. As operationally defined, high-molecular-weight material appeared in the void volume and low-molecular-weight material appeared in the end volume. The low-molecular-weight material was subsequently identified as free [^{3}H]leucine by chromatography of a fraction of absorbed sera on cellulose (Bakerflex cellulose; J. T. Baker Chemical Co., Phillipsburg, N.J.) alongside a lane containing a standard of [^{3}H]leucine.

The plastic sheet for thin-layer chromatography was developed with isopropanol-HCl-H_2O (85:22:18). The position of the radioactivity on the cellulose was determined by cutting strips (length, 1 cm) along the length of the cellulose, placing each in a scintillation vial containing 1 ml of water, shaking the vial for 1 h, and then adding 10 ml of Scintiverse II and counting radioactivity in the scintillation counter. The contents of the high-molecular-weight fraction were subsequently identified as proteins by precipitation in the cold of 0.28 ml of absorbed sera (1:100 dilution) with 60 μl of 50% trichloroacetic acid in the presence of 50 μg of RNA as carrier. After incubation at 0°C for 30 min, the precipitate was collected by centrifugation, washed twice with ethanol, air dried, and solubilized in SDS-PAGE sample buffer. The trichloroacetic acid-precipitable material was then subjected to SDS-PAGE (17) on 10% acrylamide gels, and the gel was dried and autoradiographed. In some experiments absorption was performed at room temperature for various periods of time. In others the bacteria were doubly labeled by also adding [2-^{14}C]acetate to the growth medium (0.63 μCi/ml; 54 mCi/mmol; Amersham), thus enabling us to measure both released protein and membrane lipid. ([2-^{14}C]acetate exclusively labels lipids of *H. influenzae* under these conditions [17].)

Preparations of cell fractions. Outer membranes of strain Eag for use in the immunoblot assay were prepared by breaking logarithmic-phase cells in a French press and isolating outer membranes by sucrose gradient centrifugation (17). Outer membranes for determination of OMP profiles on SDS-PAGE were prepared by an extraction procedure with Triton X-100 (17). Periplasmic proteins were obtained from 1.25×10^{10} CFU of strain Eag as described previously (21) and were analyzed by SDS-PAGE.

Statistics. The statistical significance of data was determined with Student's *t* test.

RESULTS

Stability of the *H. influenzae* cell envelope in serum. An assay for identifying surface-exposed antigens requires that the cell envelope maintain its integrity. If incubation in serum should cause the outer membrane to peel back off the surface of the cell or result in the release of unsealed outer membrane fragments, then epitopes on both the outer as well as the inner face of the outer membrane would be accessible to antibodies. Since the stability of *H. influenzae* has not been examined previously, we performed a series of experiments to assess its stability in serum.

Strain Eag, which was labeled by growing it in [^{3}H]leucine, released a small amount of its radioactivity into the medium when incubated in PCM or in a 1:100 dilution of serum (0.41 and 0.59%, respectively; Table 2) and somewhat more (1.5%; $P < 0.01$) on incubation in undiluted serum (Table 2). However, very little of this was in large-molecular-weight material (0.04, 0.09, and 0.07%, respectively; Table 2; see Fig. 1A and B); differences within this group were not significant. Most of the released radioactivity was subsequently shown to be free [^{3}H]leucine, which may represent leakage from the intracellular leucine pool or radioactivity nonspecifically associated with the bacteria and not completely removed during the washes.

Quite different results in two regards were obtained with strain S2. (i) Severalfold greater amounts of total cell-associated ^{3}H were released ($P < 0.01$ for strain Eag versus S2), regardless of whether incubation was in buffer or serum (Table 2; see Fig. 1C and D). (ii) A large part of the ^{3}H released was in large-molecular-weight material. Analysis by SDS-PAGE followed by autoradiography showed the presence of many proteins that varied in molecular weight, from 20,000 to 70,000, and that had the same gel profile as periplasmic proteins from strain S2 (data not shown). Thus, strain S2 demonstrates considerable envelope instability compared with its parent strain. These results raise the question as to whether this is due to the lack of a capsule. However, an untypable isolate (strain C353), whether incubated with homologous antibodies or with antiserum to strain Eag, gave results similar to those obtained with strain Eag (Table 2). Hence, the loss of capsule per se is not associated with instability.

Other experiments with strain Eag showed that incubation at 20°C yielded results similar to those with incubation at 0°C with regard to the release of high-molecular-weight material. However, the amount of free leucine released was higher during incubation in dilute antiserum or PCM (Table 2). Also in the single experiment, an increase in the incubation time at 0°C to 120 min resulted in two- to fourfold increases in released radioactivity in both large and small fractions (Table 2).

Further insight into the differences in stability of strains Eag and S2 in buffer and antiserum was obtained by studying release from cells doubly labeled by growth in medium containing [^{3}H]leucine and [^{14}C]acetate. Typical data on the size fractionation of the released material are given in Fig. 1A to D; note the differences in the ordinate scales. The important points involving ^{14}C release are as follows. (i) Essentially all of the ^{14}C label appeared in the large-molecular-weight fraction and, hence, represents lipid incorporated into the membrane. (ii) Strain Eag incubated in PCM

TABLE 2. Release of [^{3}H]leucine containing molecules from *H. influenzae* type b strains after incubation in PCM or antisera

Strain/antiserum	Incubation conditions (min, °C)	Total released ^{3}H cpm (as % cellular cpm) at the following serum concn[a,b]:			Released ^{3}H cpm in large-mol-wt fraction (as % cellular cpm) at the following serum concn[b]:		
		None	1:100	Neat	None	1:100	Neat
Eag/anti-Eag	30, 0	0.41 ± 0.28	0.59 ± 0.27	1.5 ± 0.28	0.04 ± 0.02	0.09 ± 0.04	0.07 ± 0.02
S2/anti-Eag	30, 0	2.2 ± 1.3	3.3 ± 0.38	4.3 ± 0.14	1.3 ± 1.2	2.0 ± 0.9	2.0 ± 0.07
C353/anti-Eag	30, 0	0.55 ± 0.12	0.69 ± 0.11	1.3 ± 0.14	0.18 ± 0.07	0.23 ± 0.03	0.33 ± 0.03
C353/anti-C353[c]	30, 0	0.67 ± 0.15	0.58 ± 0.07	1.1 ± 0.04	0.11 ± 0.06	0.17 ± 0.10	0.07 ± 0.04
Eag/anti-Eag	30, 20	1.91 ± 0.4	2.0 ± 0.5	1.77 ± 0.4	0.03 ± 0.03	0.08 ± 0.03	0.11 ± 0.03
Eag/anti-Eag	30, 0	0.39		0.42	0.01		0.04
	120, 0	1.05		1.03	0.04		0.08

[a] Total [^{3}H]leucine incorporated averaged 1.5×10^6 cpm/1.8×10^{10} bacteria.

[b] Data are expressed as mean ± standard deviation and are from two to five experiments, except for data in the last two horizontal rows, for which only one experiment was performed.

[c] Absorption was performed with a purified immunoglobulin G fraction of the antiserum (9).

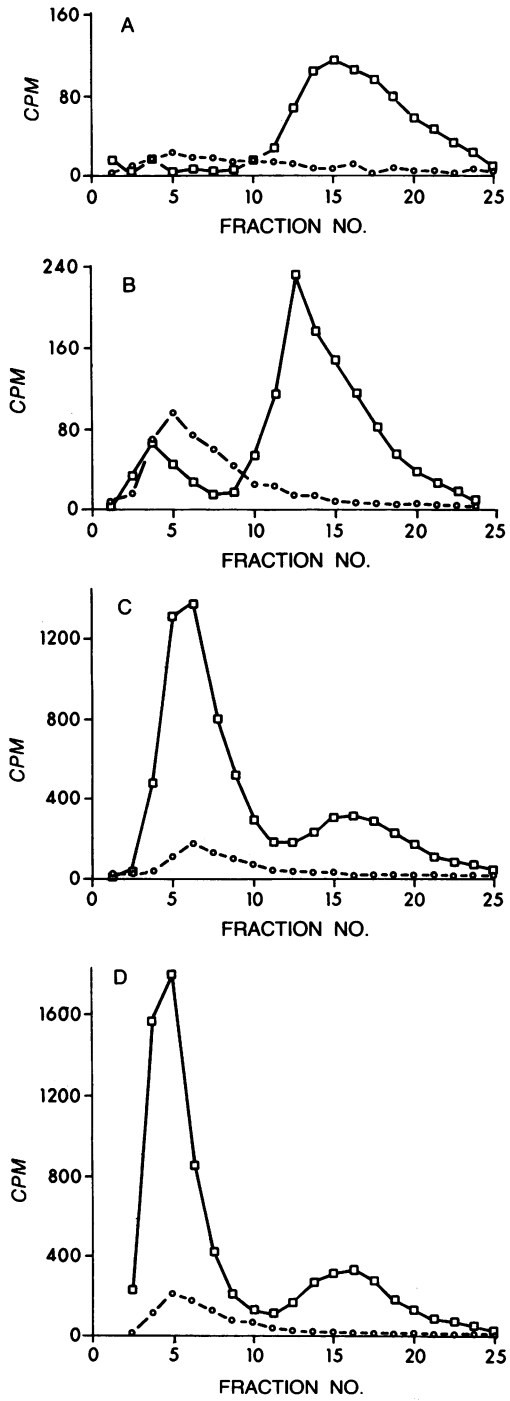

FIG. 1. Size fractionation of ^{3}H- and ^{14}C-labeled material from doubly labeled strains Eag and S2 during incubation for 30 min at 0°C in buffer (PCM) or R99 antiserum (1:100 dilution). (A) Strain Eag in PCM; (B) strain Eag in R99; (C) strain S2 in PCM; (D) strain S2 in R99. Samples were applied to a Bio-Gel P2 column, and five-drop fractions were collected. The void volume is at fraction 5. Symbols: ○, ^{14}C; □, ^{3}H.

released a very small percentage of its membrane, and this was increased about threefold by incubation in antiserum at a 1:100 dilution ($P < 0.05$; data from two experiments). In contrast, strain S2 released the same amount of membrane whether incubation was in PCM or antiserum. (iii) When incubated in antiserum (1:100 dilution), strain S2 released three times more of its membrane but 22 times more of its protein compared with strain Eag. The results with ^{3}H label (Fig. 1) present the data described above and supplied in Table 2.

Immunoblot assay for cross-reactivity of exposed surface epitopes. (i) Selection of antiserum. Different batches of rabbit antiserum to live strain Eag varied in their proportion of antibodies to exposed epitopes of individual OMPs and, hence, in their usefulness in the assay. Thus, R99, an antiserum to live strain Eag, was previously shown to be effective for detecting surfaced-exposed epitopes to protein *n* (a minor 100K protein) and to LPS (Fig. 2 in reference 13). A subsequent rabbit antiserum (R102) to the S2 mutant of strain Eag was found to be effective for protein *a* and LPS (Fig. 2). Data from experiments with these two antisera are described below.

(ii) Cross-reactivity of exposed epitopes on protein *a*. Antiserum R102 was absorbed with the *H. influenzae* strains listed in Table 1 (rows 1 to 21) and then compared with unabsorbed R102 by the immunoblot procedure. These strains represented a broad spectrum in terms of evolutionary relatedness, as indicated by ETs, which ranged from 1 to 32. Typical results obtained with these strains are given in Fig. 2. Compared with unabsorbed antiserum, antiserum absorbed with strain 1064 or strain 1079 was depleted of antibodies to protein *a*, whereas absorption with strain 1054 did not result in depletion. Therefore, strains 1064 and 1079 contain at least one surface-exposed epitope of protein *a* that is cross-reactive with protein *a* of strain Eag. In fact, since almost all of the antibodies were removed by both of these strains, the same epitope(s) is probably involved. The third strain (1054) does not possess this epitope(s). However, because only positive results are interpretable in this assay, the negative result obtained with strain 1054 does not preclude the existence of other surface-exposed cross-reactive epitopes of protein *a*.

Strains listed in Table 1 (rows 1 to 14) possess a protein *a* with the same mobility as that of protein *a* from strain Eag;

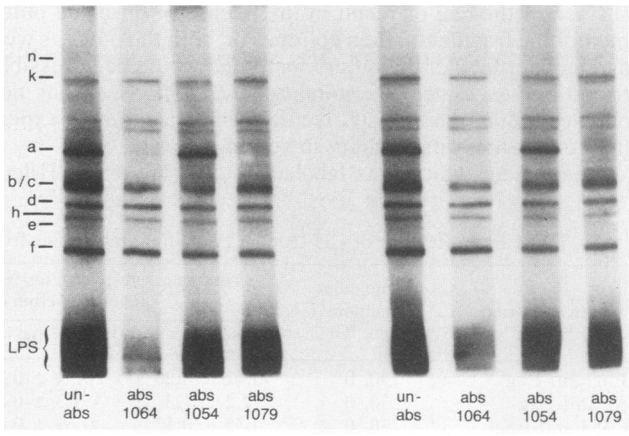

FIG. 2. Immunoblot assay for cross-reactive surface-exposed epitopes. R102 antisera were absorbed (abs) with *H. influenzae* type b, strains 1064, 1054, and 1079. The absorbed and unabsorbed (unabs) sera were then subjected to the immunoblot assay. The autoradiograph obtained with duplicate samples is shown. The letters on the left refer to the positions of the predominant OMPs and LPS. The protein positions were determined by India ink staining (6) of a companion strip of nitrocellulose.

i.e., OMP subtype is of the L group. All but one of these strains (1072) removed antibodies to protein *a* in the R102 antiserum raised to strain Eag. These 13 positive strains represent a variety of OMP subtypes, but more importantly, they come from strains that are considerably distant from each other on an evolutionary scale; i.e., ETs 4 to 24 are represented. The strains listed in rows 15 to 21 in Table 1 have a protein *a* with either faster (the U strains) or slower (the H strains) mobility than that of protein *a* of strain Eag. Only one of these strains (1063) removed antibodies to protein *a*; this strain is quite distant from strain Eag on the evolutionary scale. These data are summarized in Table 3.

(iii) Cross-reactivity of exposed epitopes on protein *n*. Protein *n* is a minor OMP with a molecular weight of approximately 100,000 and is not part of the OMP subtyping scheme. Hence any one subtype may contain strains with protein *n*'s of different mobilities on SDS-PAGE (data not shown). Therefore for the immunoblot assay with R99 antiserum, we chose strains for which we already had information on the mobility of their protein *n*'s, their OMP subtypes, and their ETs (Table 1, rows 22 to 31). Of the five strains whose protein *n* had the same mobility as that of strain Eag (Table 1, rows 23 to 26 and 31), four absorbed antibodies to protein *n* of strain Eag; the exception was strain 1148 (Table 1). In contrast, those five strains with a protein *n* of different mobility (Table 1, rows 22 and 27 to 30) did not absorb antibodies to protein *n*. Two strains with different cross-reactive specificities were in the same ET group (Table 1, rows 22 and 23). However, five of the six strains not recognized by antiserum to strain Eag (Table 1, rows 27 to 31) were in more distant ET groups. The prevalence of this evolutionary separation would require the examination of more strains. These data are summarized in Table 3.

(iv) Cross-reactivity to protein *b/c* and LPS. In the immunoblot assay with R102 antiserum, approximately 70% of the strains absorbed antibodies to protein *b/c* of strain Eag. This apparent absorption occurred with strains with protein *b/c*'s with different mobilities on SDS-PAGE and that possessed various ETs. In addition, all strains that absorbed antibodies to protein *b/c* also absorbed antibodies to LPS, and conversely, strains that did not absorb antibodies to protein *b/c* did not absorb antibodies to LPS. This correlation may be real, but we suspect that it is an artifact due to the strong affinity that LPS has for some OMPs, especially those which, like protein *b/c*, are porins (4, 23). This possibility was indicated by results of limited studies which showed that absorption with antiserum depleted of antibodies to LPS no longer appeared to remove antibodies to protein *b/c* (data not shown).

TABLE 3. Relation between ET and mobility on SDS-PAGE and the absorption of antibodies to OMPs *a* and *n* of strain Eag

Absorption properties of strains	Mobility of proteins (no. of ETs) compared with those of strain Eag proteins			
	Protein *a*		Protein *n*	
	Same	Different	Same	Different
Absorbent strains; ETs	4, 6, 8, 10, 11, 12, 12, 19, 20, 21, 22, 23, 24	27	1, 1, 1, 1	–
Nonabsorbent strains; ETs	13	1, 7, 9, 25, 30, 32	30	1, 12, 15, 17, 21

DISCUSSION

An ideal assay for assessing the cross-reactivity of surface-exposed epitopes should yield neither false-positive nor false-negative results. To date, no such assay has been described, and the assays that are used have a variety of problems. Thus, attempts to identify surface-exposed proteins of *H. influenzae* type b by methods suitable for eucaryotic cells, namely, lactoperoxidase or IodoGen (Pierce Chemical Co., Rockford, Ill.)-catalyzed iodination of proteins with ^{125}I, failed (16), as indicated by the labeling of both inner membrane proteins and OMPs.

In an alternate assay, whole-cell radioimmunoprecipitation (7), radioactively labeled *H. influenzae* was incubated with antibodies and then solubilized with a detergent cocktail with the intent of yielding discrete immune complexes, which were then collected by absorption onto protein A-bearing *Staphylococcus aureus* and then identified by SDS-PAGE and autoradiography. In some cases, however, false-positive results were obtained because of incomplete dissociation of all outer membrane components by the detergent. Thus, early reports that a 39K OMP of *H. influenzae* type b was surface exposed and induced protective antibodies (7, 8) were subsequently shown to be due to coprecipitation of an undissociated complex of the labeled 39K protein and unlabeled LPS by antibody that was actually specific for the LPS (4).

The immunoblot assay was developed as an alternative to whole-cell radioimmunoprecipitation. A positive result is always meaningful, provided that the bacterium maintains its integrity during absorption. To be effective, however, several conditions are required. Antigen (bacteria) must be present in excess during absorption, the serum must contain surface-specific antibodies in sufficient amounts relative to total antibody, and the surface-exposed regions of the antigens on the nitrocellulose must retain their native structure, in spite of having gone through SDS-PAGE and electroblotting. The first of these conditions is easy to control, but the other two are not. Hence, a critical feature of this assay is that a negative result cannot be interpreted. In addition, we have not been able to identify a single serum that can be used for all of the predominant *H. influenzae* antigens.

Finally, a modification of the immunoblot procedure has been described previously (2). Rather than examining the serum after absorption, the absorbed antibodies were eluted off the bacteria by treatment with glycine buffer at pH 2.8 and then assayed by immunoblotting. In our hands, using absorption conditions suitable for *H. influenzae*, we observed that this method is considerably less sensitive than the immunoblot assay (data not shown).

In all of the methods described above, it is critical that the integrity of the *H. influenzae* outer membrane be maintained throughout the procedure. Experiments demonstrated the stability of the strain Eag envelope, provided that absorption was performed at 0°C for 30 min on logarithmic-phase cells used immediately after harvest. Strain S2, in contrast, was quite unstable and, thus, is not a good control for the role of capsule in clearance (24), in virulence, or, as reported previously (13), in hindering access of antibodies to OMPs. It should be noted that strain S2, which has the same outer membrane and LPS profiles as strain Eag on SDS-PAGE (11, 14), is a double mutant. The first mutation is selection for streptomycin resistance and the second is for spontaneous loss of capsule (18, 24). Neither of these mutations has been analyzed genetically, precluding further insight into the cause of envelope fragility.

The outer membrane released from strain Eag could represent the release of much material from a few cells or very small amounts of material from many cells. We propose the latter type of release since significant loss of outer membrane would also result in the release of nonmembrane proteins (e.g., periplasmic proteins) and since the protein released from strain Eag is essentially membrane protein. This is also supported by absorption data. Whereas strain S2 removes antibodies to many outer membrane components, strain Eag is more restricted (Fig. 2 in reference 13). Since other *H. influenzae* type b strains behaved like strain Eag in the absorption experiments reported here, it is implied that they also have stable envelopes, although this was not directly measured for other type b strains.

Having now discussed the methods and their limitations for measuring cross-reactivity of surface-exposed epitopes, we briefly focus on the data regarding proteins *a* and *n*. For each protein we have demonstrated that considerable but limited cross-reactivity exists.

Regardless of their evolutionary distance, 13 of 14 strains that possessed a protein *a* with the same mobility as that of protein *a* of strain Eag shared surface-exposed cross-reactive epitopes with protein *a* of strain Eag. There was only one exception to this, strain 1072, which possessed ET13 and OMP subtype 3L. The possibility that other strains of the L OMP subtype from ET13 possess this property has yet to be examined. However, another 3L strain (1071) does not possess this property; i.e., it did recognize antibodies to protein *a* of strain Eag. Of seven strains with a different protein *a* from that of strain Eag, one (1063) possessed cross-reactive epitopes. Thus, these data reveal a significant finding, namely, that one protein *a* (from strain 1072) with the same mobility as that of protein *a* of strain Eag differs biochemically in exposed regions of the molecule, and also that one protein *a* (from strain 1063) with a different mobility has biochemical similarities with strain Eag in the exposed regions of the molecule.

Although the immunoblot assay indicated that strains of the H OMP subtype do not share cross-reactive epitopes with strain Eag, protection data indicated otherwise (13a); i.e., antibodies to purified protein *a* of strain Eag protect infant rats from infection by four strains tested, two of which possess the H OMP subtype (one of these H strains, 1054, was used in the experiments described above). This result serves to reinforce the proviso that only positive data are meaningful in the immunoblot assay. Apparently, H strains can possess cross-reactive epitopes. Since the protective antiserum specific for protein *a* also tests negative in the immunoblot assay when absorbed with H strains, we suspect that the cross-reactive epitopes common to H and L strains do not survive the immunoblot assay procedure because of the denaturability of their chemical structure. This result highlights the difficulties in readily detecting cross-reactive epitopes. Yet such information is highly important for developing OMP vaccines.

The data with protein *n* are of interest because evolutionary distance appears to play a role in the cross-reactivity of exposed epitopes. Thus, of the 10 strains examined, 6 did not remove antibodies to protein *n* of strain Eag, and all but 1 of these were ET12 or higher, including 1 strain (1148) that had a protein *n* with the same mobility. Thus, again, as with protein *a*, similarity of mobility does not mean biochemical identity. This interesting, but not completely exclusive, evolutionary clustering of diverse forms of protein *n* requires confirmation by examination of additional strains.

The findings presented above are important for understanding evolutionary pressures on the exposed surfaces of OMPs and for identifying suitable vaccine candidates.

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