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Lactoperoxidase and Iodo-Gen-Catalyzed Iodination Labels Inner and Outer Membrane Proteins of *Haemophilus influenzae*

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Both inner and outer membrane proteins of *Haemophilus influenzae* type b were labeled by iodination procedures believed to be specific for exposed surface proteins only. It is suggested that this is due to specific properties of the outer membrane of *H. influenzae* and that use of these procedures with other gram-negative bacteria be evaluated carefully.

In the course of determining the role of outer membrane proteins of *Haemophilus influenzae* in the pathogenesis of disease caused by this bacterium, we attempted to identify those cell surface proteins (i.e., outer membrane proteins) that are exposed to the environment, as opposed to being buried in the membrane or occluded by capsule or lipopolysaccharide. We used two methods which had been developed for their ability to specifically label the surface proteins of eucaryotic cells (8, 12) with ^{125}I and have since been used for labeling the surfaces of gram-negative bacteria (e.g., references 4, 5, 7, and 9), including *H. influenzae* (2, 3), albeit with little critical evaluation of these methods for bacteria.

In the first method, cells were incubated with lactoperoxidase, $Na^{125}I$, and H_2O_2; the lactoperoxidase catalyzes the oxidation of H_2O_2 which then generates the active iodinating species (10). In the second method, the cells and $Na^{125}I$ were added to tubes which had been coated with the water-insoluble catalyst, 1,3,4,6-tetrachloro-3α,6α-diphenylglucoruril (Iodo-Gen; Pierce Chemical Co., Rockford, Ill.) (8). However, in our hands, use of either of these methods resulted in labeling the inner as well as the outer membrane proteins of *H. influenzae*, a surprising outcome that casts doubt on the vectorial specificity of these methods, at least with this bacterium. In this report, we present the details of our experiments and discuss why these methods of surface labeling are ineffective with *H. influenzae*, and possibly, other gram-negative bacteria.

As part of the background, it should be noted that the cell envelope of gram-negative bacteria can be described functionally and structurally as consisting of an inner (cytoplasmic) membrane, a matrix of peptidoglycan (murein), a periplasmic space, and an outer membrane, the last being the outermost layer of the cell (1). In addition to these required components, the surface may also contain pili, flagellae, or capsule. *H. influenzae* possesses this typical overall envelope structure (6, 13, 17). Furthermore, its outer membrane is also typical in that it contains phospholipid, lipopolysaccharide, and about 24 proteins, 6 (major outer membrane proteins) of which account for about 75% of the total protein (6).

In our experiments, *H. influenzae* type b was labeled in vivo by growth in medium containing [2-^{14}C]acetate, which specifically labels the lipids (i.e., membranes) (6), and were then labeled in vitro with ^{125}I by the procedure of Hansen et al. (3). Specifically, *H. influenzae* type b strain Eag were grown in 250 ml of brain heart infusion medium containing 10 μg of hemin per ml, 2 μg of NAD per ml, and 100 mCi of [2-^{14}C]acetate (40 to 60 mCi/mmol, Amersham Corp., Arlington Heights, Ill.). The cells were harvested at a cell density of 5×10^8/ml and washed twice in 0.01 M sodium phosphate–0.14 M NaCl (PBS). All further procedures were performed at 0 to 8°C. The cells were suspended in 3.3 ml of PBS, and to a 2-ml portion (7.6×10^{10} cells), 2 nmol of KI and 0.5 mCi of ^{125}I (obtained as carrier free $Na^{125}I$ from New England Nuclear Corp., Boston, Mass.) were added at zero time, and 50 μg of lactoperoxidase (60 to 80 U/ml; Sigma Chemical Co., St. Louis, Mo.) and 50 μl of 0.03% H_2O_2 were added at 0, 4, 8, and 12 min. The reaction was stopped at 16 min by the addition of sodium azide to 4.5 mM, and the cells were then combined with the remaining noniodinated 1.3 ml of cells, added to PBS–0.025 M NaI, collected by centrifugation, and washed twice in the buffer. The cell pellet was frozen. The following day the pellet was defrosted, brought to a total volume

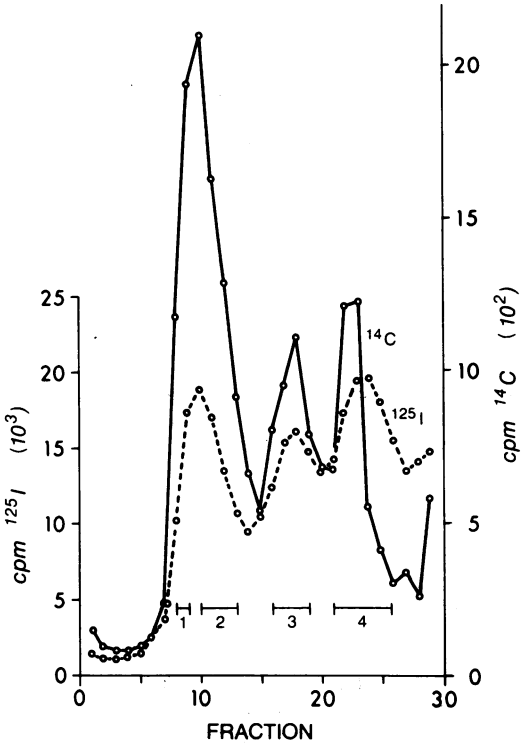

FIG. 1. Isopycnic centrifugation of cell lysates of *H. influenzae* doubly labeled with ^{14}C and ^{125}I. The bottom of the gradient is on the left.

of 6 ml with 0.05 M Tris-hydrochloride (pH 7.8), and disrupted by passage through a French pressure cell (6). After the removal of unbroken cells by low-speed centrifugation, the cell lysate was layered onto two linear sucrose gradients (6) and centrifuged in an SW27 rotor for 65 h. Fractions (ca. 1 ml) were collected from the bottom of the tube. To determine the distribution of ^{14}C and ^{125}I across the gradient, 50 μl of each fraction was added to 1 ml of water in a scintillation vial; 10 ml of Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.) was added, and the counts per minute were determined with a Packard scintillation counter (Model 3375; Packard Instrument Co., Inc., Downers Grove, Ill.) by use of appropriate settings on the red and green channels. Figure 1 shows the distributions of ^{14}C and ^{125}I on the sucrose gradient.

The ^{14}C and ^{125}I appeared in three peaks. In the two heavier peaks both labels appeared in material of the same bouyant density, whereas in the third peak the ^{125}I-labeled material was lighter than the ^{14}C-labeled components. The three peaks can be segregated into four fractions, as indicated by the numbers 1, 2, 3, and 4, near the bottom of the figure. Fraction 1 consists of outer membrane; fraction 2 consists of outer membrane with some inner membrane contamination; fraction 3 consists of inner membrane; fraction 4, an anomaly, appears to be derived from the inner membrane (6).

The finding of ^{125}I throughout the gradient instead of in the first peak suggests that components other than outer membranes were labeled. However, since inner membranes usually are contaminated with outer membranes, it was necessary to examine the distribution of ^{125}I in the proteins of each peak. Tubes representing each fraction were pooled (as marked on the figure), and centrifuged at 215,000 × *g* for 3 h. The pellets were then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (6). The protein composition of each fraction as seen on a stained gel appears in Fig. 2A. Pertinent to this discussion are lanes 1 and 3. The outer membrane (fraction 1, lane 1) consists of about 24 proteins, with the major proteins labeled a, b, c, d, e, and f. (Proteins b and c travel very close together on the gel and can be distinguished only when smaller amounts of outer membrane are applied. Also, many minor proteins are not apparent in this reproduction.) The inner membrane (fraction 3, lane 3) contains a distinctive set of proteins as well as some outer membrane proteins as contaminants. The highly stained background, especially in the upper part of the gel, is typical of our inner membrane preparations. It may represent the large number of different minor proteins present in this complex multifunctional membrane or reflect the

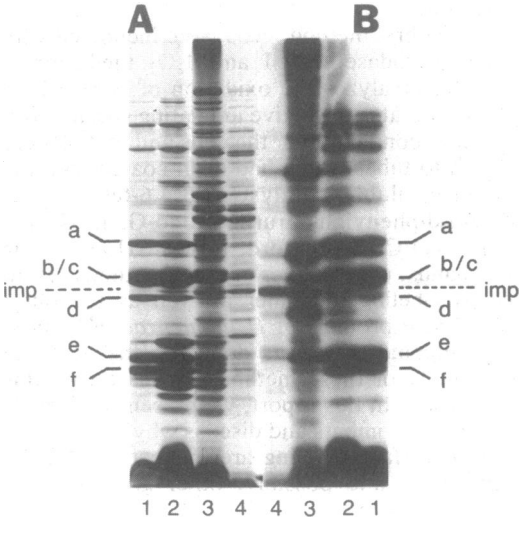

FIG. 2. SDS-polyacrylamide gel electrophoresis of membrane fractions. (A), stained gel; (B), autoradiograph of stained gel. Fractions 1, 2, 3, and 4 appear in lanes 1, 2, 3, and 4, respectively. Approximately 20 μg of protein were applied to each lane.

propensity of some inner membrane proteins to aggregate when heated in SDS (15). One of the inner membrane proteins, labeled imp, migrated between proteins b/c and protein d; it was nearly absent in fraction 1, more obvious in fraction 2, but more prevalent in fractions 3 and 4. This protein can be used as a marker for inner membrane.

An autoradiogram of the stained gel reveals the identity of ^{125}I-labeled proteins (Fig. 2B). Clearly, inner membrane proteins (lane 3) as well as outer membrane proteins (lane 1) were labeled. Thus, protein imp increased in intensity in fractions 3 and 4; the autoradiograph of fraction 3 displayed greater background than that of the other fractions, a feature typical of inner membrane, as noted above.

Thus, these data clearly show that use of published methods for labeling the surface proteins of *H. influenzae* resulted in significant labeling of inner membrane proteins. In fact, the specific activity (3.4×10^6 and 2.4×10^6 cpm per mg of protein, respectively) was approximately the same for fractions 1 and 3, a finding that is also apparent from a comparison of the intensity of the autoradiographs of lanes 1 and 3; these were similar in intensity, and equal amounts of protein were applied to the gel.

Very similar results were obtained with the use of Iodo-Gen as catalyst, although the total ^{125}I incorporated was much less (1.0×10^{-4} versus 14×10^{-4} cpm per cell). In these experiments, the *H. influenzae* (7.6×10^{10} cells in 2 ml of PBS) and 0.5 mCi of Na^{125}I were added to tubes coated with 4 μg of Iodo-Gen; carrier I^- was omitted, and the reaction was performed for 16 min at 0°C.

The failure of either of these two methods to label only the outer surface of *H. influenzae* prompted us to consider the possible reason for these results. Iodination by lactoperoxidase is surface specific presumably because the enzyme, which is too large to pass through the membrane, forms a complex on the surface of the cell with its substrate, surface-exposed tyrosine residues, and passes the iodinating species generated via enzyme action on H_2O_2 directly to tyrosine (10). However, critical investigations of this method, as used with eucaryotic cells, show that several factors must be carefully controlled to restrict labeling only to the cell surface (10, 16). In particular, the concentrations of I^-, enzyme, and H_2O_2 must be kept low relative to substrate; otherwise, free iodinating species (e.g., I_2, I^+, I_3^-), which can pass through the membranes, will be formed. Similar precautions must be used in the Iodo-Gen-catalyzed reaction since vectorial labeling in this case depends on the surface-exposed substrate being in excess over the iodinating species generated, on the rate of iodination being greater than the rate of diffusion of the iodinating agent though the membrane, and on the restriction of the catalyst to the walls of the reaction vessel (8). We did try different labeling conditions, especially those which would minimize the formation of excess free iodinating species. In these experiments, ^{125}I-labeled cells, either of strain Eag or an unencapsulated variant, were directly subjected to SDS-polyacrylamide gel electrophoresis, and protein imp was used as a marker for inner membrane. However, none of the following was successful in eliminating significant amounts of label from inner membrane: 0.1 as much Iodo-Gen, 0.04 as much H_2O_2, very fresh $^{125}I^-$, a shorter reaction time of 1.5 min, higher temperatures (22 and 37°C), absence of carrier I , and use of glucose and glucose oxidase to generate H_2O_2. In addition, control experiments showed the requirement for catalyst. In the absence of lactoperoxidase (with or without H_2O_2), labeling was reduced by more than 98%; in the presence of lactoperoxidase (without H_2O_2) labeling was reduced by more than 93%. These results show that iodination catalyzed by cellular enzymes contributed minimally to the results.

It is of interest that by using the Iodo-Gen procedure with a reaction time of only 1.5 min, the same labeled protein profile was obtained as with a 10-min incubation, although only 15% as much label was incorporated. This can be compared to results recently reported on iodination of *Neisseria gonorrhoeae* (14). These authors also found that the lactoperoxidase procedure was not surface specific. However, using the Iodo-Gen procedure and a very short reaction time of 45 s, they were able to restrict labeling to outer membrane proteins. Thus, it is possible that conditions even more stringent than those we tried might be specific for the cell surface of *H. influenzae*. However, if vectorial labeling can only be achieved under highly restrictive conditions, then its usefulness for identifying those outer membrane proteins that are surface exposed is questionable, since, temporally, it would be difficult to find precise conditions for labeling exposed tyrosines as compared to tyrosines that are buried in the outer membrane.

Hence, unlike eucaryotic cells, conditions for specifically labeling the surface of *H. influenzae* would at best be difficult to define. We suggest that this is due to the particular properties of the outer membrane which, in other gram-negative bacteria, have been shown to be covered with pores that enable passive diffusion of many small molecules (11). It is likely that *H. influenzae* also has pores. Thus, we assume that regardless of attempts to limit formation of free iodinating species, sufficient amounts are made and readily pass through the pores where reaction

with inner membrane proteins occurs. It is also possible that the surface of *H. influenzae* has very few free tyrosines available for iodination. If so, then for reasons mentioned above, labeling of inner membrane proteins would be inevitable. Finally, although electron micrographs of *H. influenzae* reveal a continuous outer membrane surrounding the cell (6), it is possible that this barrier is briefly disrupted under certain conditions, thereby allowing iodination of inner membranes. We can only offer all of the above as suggestions, since we have not performed experiments aimed at elucidating the mechanism of iodination of nonexposed proteins.

It is very likely, however, that iodination stops at the outer surface of the inner membrane. This would explain why not all inner membrane proteins are labeled with ^{125}I and why some have disproportionate amounts of the label (cf. both lanes 3, Fig. 2). Furthermore, Concino and Goodgal (2) found that exposing *H. influenzae* to low concentrations of I^- and Iodo-Gen labeled primarily the cell envelope, with minimal label appearing in the cytoplasm. (In these experiments labeling of outer versus inner membrane was not determined.)

In summary, we found that conditions suitable for specifically labeling the surface of eucaryotic cells were not applicable to *H. influenzae*. We suggest this is due to particular properties of the outer membrane. Although both methods of iodination have been used with several gram-negative bacteria, the extent of labeling of inner membranes has rarely been examined. Therefore, in the cited work with *H. influenzae* (2, 3), and possibly with other gram-negative bacteria, conclusions based on data obtained with these methods may need to be reevaluated. Furthermore, since a few individual outer membrane proteins make up the bulk of envelope proteins, labeling of inner membranes may be overlooked in examining autoradiographs obtained from SDS-polyacrylamide gel electrophoresis of whole ^{125}I-labeled cells, unless the inner membranes themselves are isolated and examined.

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