THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 44, pp. 36657–36663, November 4, 2005
Printed in the U.S.A.

Binding of *Escherichia coli* Hemolysin and Activation of the Target Cells Is Not Receptor-dependent*

Received for publication, July 15, 2005, and in revised form, August 30, 2005 Published, JBC Papers in Press, August 30, 2005, DOI 10.1074/jbc.M507690200

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Production of a single cysteine substitution mutant, S177C, allowed *Escherichia coli* hemolysin (HlyA) to be radioactively labeled with tritiated *N*-ethylmaleimide without affecting biological activity. It thus became possible to study the binding characteristics of HlyA as well as of toxin mutants in which one or both acylation sites were deleted. All toxins bound to erythrocytes and granulocytes in a nonsaturable manner. Only wild-type toxin and the lytic monoacylated mutant stimulated production of superoxide anions in granulocytes. An oxidative burst coincided with elevation of intracellular Ca^{2+}, which was likely because of passive influx of Ca^{2+} through the toxin pores. Competition experiments showed that binding to the cells was receptor-independent, and preloading of cells with a nonlytic HlyA mutant did not abrogate the respiratory burst provoked by a subsequent application of wild-type HlyA. In contrast to a previous report, expression or activation of the β_2 integrin lymphocyte function-associated antigen-1 did not affect binding of HlyA. We conclude that HlyA binds nonspecifically to target cells and a receptor is involved neither in causing hemolysis nor in triggering cellular reactions.

Hemolysin (HlyA)[3] is a major virulence factor of *Escherichia coli* strains that cause extraintestinal infections. Similar to other members of the RTX family, the toxin binds to target cells and protein-free liposomes and forms transmembrane pores (1, 2). The toxin requires post-translational fatty acylation of two lysine residues (Lys-564 and Lys-690) in order to acquire permeabilizing activity (3). Toxin mutants in which these lysine residues are replaced with arginine are totally nonhemolytic but retain their capacity to bind to erythrocytes (4, 5) and to liposomes (6).

Many attempts have been made to delineate the mode of binding of HlyA to membranes, but the results have proven difficult to accommodate in a single model. Artificial membranes are efficiently permeabilized (7, 8), and initial binding studies indicated that erythrocytes (9) and granulocytes (10) bound the toxin in a nonsaturable manner. In contrast, binding of HlyA to erythrocytes in a saturable manner was reported (11). Subsequently, the lymphocyte function-associated antigen (LFA-1) (CD11a/CD18; $\alpha_L\beta_2$ integrin), was reported to serve as the receptor for HlyA and A*ctinobacillus actinomycetemcomitans* leukotoxin on polymorphonuclear neutrophils (PMNs) (12). This conclusion was based on the following observations: use of monoclonal antibodies to LFA-1 inhibited binding of HlyA and leukotoxin to cells; immobilized leukotoxin bound LFA-1; expression of LFA-1 in a cell line that normally lacked the integrin and was insensitive to the toxins rendered these cells sensitive to HlyA and leukotoxin. The authors suggested that nonspecific absorption of HlyA to various cell types might additionally occur that could obscure the receptor-mediate interaction (12). Studies with another RTX toxin, the leukotoxin of *Mannheimia hemolytica*, indicated that binding to integrin on bovine leukocytes results in activation of the tyrosine kinase signaling cascade (13). Binding of collagen to LFA-1 activates this signaling cascade and triggers the respiratory burst in human neutrophils (14). Because HlyA also stimulates the respiratory burst in these cells (15–17), a unifying concept would be that *E. coli* hemolysin binds to integrin LFA-1, activating the tyrosine kinase signaling cascade and triggering the respiratory burst. Binding to erythrocytes must occur via other mechanisms, however, because these cells do not express LFA-1. According to one report, glycophorin might serve as the receptor for HlyA in these cells (18). Such a concept contradicts the nonsaturable binding previously reported.

All previous investigations on the binding of RTX toxins to cells suffered from the drawback that radioactive toxin tracers were not available for quantitative measurements of binding. Several studies employed crude culture supernatants or cell sonicates rather than purified toxin preparations, and quantification of binding inevitably relied on indirect methods. In this study, we devised a method for radioactive labeling of HlyA and conducted experiments to test the hypothesis that the toxin binds to a receptor. Our results indicate that HlyA does not interact with a receptor on granulocytes. Binding occurs in a nonspecific and nonsaturable manner, and the respiratory burst is triggered directly by pore formation, probably because of flux of extracellular Ca^{2+} into the cells.

MATERIALS AND METHODS

Expression of hlyA, Mutagenesis, and Toxin Purification—Mutants in which Lys at position 564 and/or 690 was replaced by Arg (3) were kindly provided by Dr. C. Hughes. Mutation of Ser-177 into Cys was described previously (19). The same procedure was followed starting with K564R, K690R and K564R/K690R, to form S177C/K564R, S177C/K690R, and S177C/K564R/K690R, respectively. Protein purification of hemolysin was carried out as described previously (4, 19).

Labeling of Toxin—Mutant toxin containing Cys-177 was labeled with *N*-ethylmaleimide (NEM) using the following protocol. Alcohol precipitation of toxin was carried out to remove dithiothreitol as described previously (19). The precipitate was dissolved in 8 M guanidine HCl, pH 8.0, and 2 μM toxin was incubated with 50 μM NEM for 1 h

* This work was supported by Deutsche Forschungsgemeinschaft 490, Project C1/D3. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] Supported by the Intramural Research Program, NCI, National Institutes of Health.

[3] The abbreviations used are: HlyA, hemolysin; RTX, repeat in toxin; LFA, lymphocyte function-associated antigen; PMN, polymorphonuclear neutrophil; NEM, *N*-ethylmaleimide; HBSS, Hanks' balanced salt solution; WT*, tritiated wild-type S177C.