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Detoxification of the *Helicobacter pylori* Cytotoxin

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Treatment of the *Helicobacter pylori* vacuolating cytotoxin with very low concentrations of formaldehyde resulted in abrogation of toxic activity in both a HeLa cell vacuolation assay and an in vivo assay of gastric epithelial damage. Detoxification had only a minimal effect on the integrity of the oligomeric or monomeric structure. The toxoid retained the ability to bind to target cells and to induce high-titer neutralizing antibodies after immunization of rabbits. Furthermore, oral immunization of mice with the toxoid resulted in protection against infective challenge with mouse-adapted strains of *H. pylori*. The sensitivity of the toxin to formaldehyde treatment suggests that a few lysine residues in the protein may be essential for toxic activity and that VacA detoxified in this manner may be a potential candidate for inclusion in a vaccine against *H. pylori* infection and disease.

Chemical detoxification of proteins for use in human vaccines was pioneered by Ramon in 1924 (15) when he discovered that addition of formalin to culture supernatants of *Corynebacterium diphtheriae* not only stabilized the toxin from degradation but also rendered it nontoxic. Since then, this simple reaction has found widespread use in vaccine production. The basis of formaldehyde cross-linking is the rapid condensation between an amino group in the protein and the carbonyl moiety to form an imine, followed by a slow addition of a second group to the protonated imine (11). In proteins, the ε-NH_2 group of lysine residues is by far the most reactive (7), so that cross-linking generally occurs by the formation of methylene bridges between lysil residues or between lysil residues and glutamine, asparagine, arginine, or tyrosine.

One problem with this approach, however, is that extensive inter- and intramolecular cross-linking in the protein can lead to structural distortions which destroy important protective epitopes, making the detoxified molecules less immunogenic than the untreated protein (1, 12). In vaccine detoxification, the reaction is carried out in the presence of excess free lysine, which limits inter- and intrapolypeptide cross-linking to groups which are relatively closely associated in the molecule and prevents reversion of the reaction. We have used this approach to detoxify the vacuolating cytotoxin (VacA) from *Helicobacter pylori* for use in a potential vaccine against peptic ulcer disease.

The vacuolating cytotoxin produced by *H. pylori* plays a key role in gastroduodenal disease (19) and is a strong candidate for inclusion in a vaccine. Oral immunization of mice with purified cytotoxin confers protection against infection by toxin-producing strains of *H. pylori* (10). However, the cytotoxicity causes acute gastric epithelial erosion (19) and ulceration, thus excluding the active toxin from use in humans. The active toxin is a highly organized oligomeric molecule consisting of either six or seven copies of a 95-kDa polypeptide (8). Each monomer is folded into two distinct subunits which are connected by an exposed loop of a repeat of 8 amino acids (19). After release from the bacteria, this loop is cleaved specifically without altering the oligomeric structure of the protein. Hence, the toxin structure shares features with the structure of the di-chain class of bacterial toxins which includes the *Clostridia* neurotoxins, the cholera and *Escherichia coli* enteric toxins, and pertussis toxin.

Previous experience in the development of vaccines against disease caused by these toxins has shown that protective immunity is considerably more effective if the antigenic structure of the protein antigens is preserved (12, 17). This is likely to be true also for the *H. pylori* toxin, since antisera from rabbits immunized with the native toxin effectively inhibited toxic activity in vitro, whereas antisera raised against recombinant inactive 95-kDa polypeptide, which failed to oligomerize, was ineffective (9).

We have subjected highly purified VacA to treatment with different concentrations of formaldehyde and demonstrated a dose-dependent inactivation. Toxic activity was found to be extremely sensitive to formaldehyde treatment, so that conditions which resulted in complete inactivation with minimal loss of antigenicity could be found. The results open the way to the initiation of human clinical trials of the toxin as a component of an anti-*H. pylori* vaccine.

MATERIALS AND METHODS

VacA purification and detoxification. VacA was purified, as previously described (9), from culture supernatant of *H. pylori* CCUG17874. Formaldehyde treatment was carried out by incubation of VacA (approximately 100 μg/ml) in a solution of phosphate-buffered saline (PBS) containing 25 mM lysine and 0.01% thimerosal (Sigma Chemicals, St. Louis, Mo.) plus different concentrations of formaldehyde for 48 h at 37°C followed by dialysis against PBS. Control VacA was treated in the same manner but in the absence of formaldehyde. VacA biological activity was assessed in a HeLa cell-vacuolating assay (14). Briefly, 10^4 HeLa cells/well were seeded into 96-well flat-bottomed microtiter plates. After 16 h of incubation, the cells were treated for a further 8 h at 37°C with 2 μg of acid-activated VacA (2) in 100 μl of RPMI medium containing 2% fetal calf serum plus 5 mM ammonium chloride. Vacuolation was assessed by the uptake of neutral red dye. To ensure that all components of the formylation reaction were removed by dialysis, the dialysate of a dummy reaction mixture containing all components except VacA was added to untreated VacA in the vacuolation assay. No effect on VacA activity was observed.

Biochemical analysis. VacA integrity was assessed by analytical gel filtration on Superose 6 medium with a SMART system (Pharmacia, Uppsala, Sweden) and by direct visualization of the protein in transmission electron microscopy after quick-freeze deep etching (6). The extent of intermonomer cross-linking of the formylated VacA was estimated by immunoblotting the material with anti-VacA-specific antisera (6) after separation by completely denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To assess the stability of the toxoid, formaldehyde (1.6 mM)-treated VacA was incubated at 37°C in PBS for 7 days and then compared with freshly treated material in the above assays.

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Immunization of rabbits and neutralization of toxin. New Zealand White rabbits of approximately 2.5 kg were immunized by intradermal injection of four doses on days 0, 14, 28, and 56 of 25 μg each of purified toxin or formaldehyde-treated toxin in a solution of 1-mg/ml aluminum hydroxide as an adjuvant. Immunoglobulins were purified with protein G-Sepharose (Pharmacia) and tested for their ability to neutralize VacA cytotoxic activity in the in vitro vacuolation assay (14), in which serial dilutions of the purified immunoglobulins were incubated with 2 μg of purified toxin in 40 μl of PBS added to the cells in 160 μl of culture medium containing 5 mM ammonium chloride. The concentration of immunoglobulin which resulted in 50% inhibition of activity was determined directly from graphs of inhibition versus concentration, and standard deviation was calculated from the average variation of the data in each set.

In vivo toxicity. Groups of three 5-week-old male BALB/c mice were treated intragastrically with 5 μg of native or formaldehyde (3.2 mM)-treated VacA as previously described (19). The preparations were exposed to low pH in vitro in order to obtain optimal activation (2). Mouse stomachs were prepared for histology and hematoxylin and eosin staining by standard procedures. Three slides per mouse were stained and coded by one of us (P. Ghiara) and examined by another of us (M. Marchetti) who was unaware of the treatments and the code. Epithelial damage scores were given according to previously published criteria (5).

Prophylactic vaccination. Intragastric vaccination was performed as previously described (10). Briefly, groups of 10 5-week-old male CD1 specific-pathogen-free mice were treated intragastrically with three weekly doses of saline alone or saline containing 100 μg of formaldehyde (1.6 mM)-treated VacA per dose plus 10 μg of *E. coli* heat-labile toxin per dose as an adjuvant. One week after the last dose, the mice were challenged with 10^9 CFU of the mouse-adapted *H. pylori* strain SPM326. Infection was assessed 2 weeks after challenge by culture of bacteria from the mouse stomachs (10).

Electron microscopy. Native or formaldehyde-treated VacA was prepared for quick-freeze, deep-etch electron microscopy (6) after absorption to mica flakes (8). Frozen mica flakes were freeze fractured in a Balzers Baf 301 freeze-etching unit and etched for 4.5 min at −104°C. Molecules absorbed to the mica were rotary replicated with an approximately 2-nm-thick layer of platinum applied from an angle of 10^0 above the horizontal and then backed with a 25-nm-thick film of pure carbon. Replicas were viewed in a Philips CM10 transmission electron microscope operating at 80 kV.

Cell binding. HeLa cells grown in RPMI medium containing 10% heat-inactivated fetal calf serum were washed and treated with 10 μg of formaldehyde-treated VacA per ml for 1 h at 0°C. After being washed with PBS, the cells were further treated with 8 μg of purified immunoglobulin per ml from a rabbit serum raised against native VacA. Cell-bound VacA was revealed by flow cytometry in a FACScan (Beckton Dickinson) with fluorescein isothiocyanate-labelled anti-rabbit immunoglobulin antibodies.

Statistics. Unless otherwise stated, the Student *t* test was used for statistical evaluation of the data.

RESULTS

Highly purified oligomeric cytotoxin was subjected to treatment with different concentrations of formaldehyde in the presence of excess free lysine, and toxicity was assessed in a HeLa cell vacuolization assay (Fig. 1). The dose response revealed that VacA biological activity was extremely sensitive to formaldehyde treatment. With 0.8 mM formaldehyde, toxic activity was reduced by 90%, and with 1.6 mM formaldehyde, the protein was completely detoxified. At this concentration, the free lysine (25 mM) in the reaction mixture was in 16-fold molar excess. Hence, at reaction completion, a maximum of 12.5% of the total lysine could be modified, since each formaldehyde molecule can cross-link two lysine molecules. Given the limited reaction and the preference for lysine residues, the data suggest that a small number of the 50 lysine residues in the VacA monomer are highly susceptible to formaldehyde cross-linking and that these residues may be important for biological activity.

Formylated VacA was also found to be inactive in an in vivo mouse model of toxin-induced pathology. It has been shown previously that intragastric administration of highly purified toxin to mice causes gastric epithelial erosion and ulceration similar to those observed in *H. pylori*-associated pathologies in humans (19). Treatment with formaldehyde (1.6 mM) rendered the toxin essentially inactive in this model (Fig. 2). Hence, the in vivo toxicity of the protein is directly attributable to its cell toxicity, as measured in the in vitro HeLa cell assay.

In analytical gel filtration, the untreated toxin eluted as a single uniform peak of approximately 700 kDa. The majority of the formylated toxin eluted in a uniform peak at the same volume, but a small peak of material which eluted earlier and was likely to be a small quantity of aggregated oligomers was detected (Fig. 3). Hence, the formaldehyde treatment did not result in extensive degradation of the protein, and the presence of the excess free lysine effectively prevented extensive interoligomer cross-linking. However, cross-linking between the 95-kDa monomers could be detected after SDS-PAGE and immunoblotting with toxin-specific antisera. Before treatment with formaldehyde, immunoblotting of the toxin revealed the 95-kDa monomer (Fig. 4, lane 5). At the lowest concentration of formaldehyde, little appreciable difference from the untreated sample could be observed. At higher formaldehyde concentrations, immunoreactive material which migrated more slowly than the 95-kDa polypeptide was found, indicating significant intermonomer cross-linking. At the highest formaldehyde concentration, the majority of the toxin did not enter the

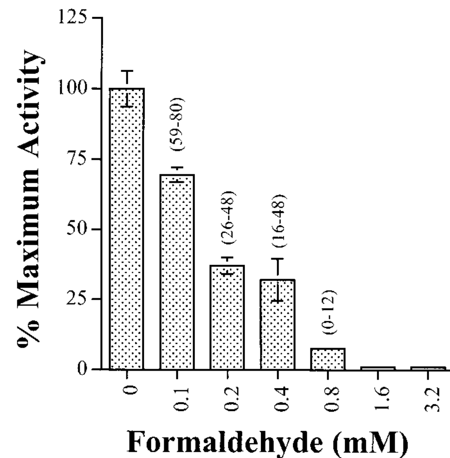

FIG. 1. Vacuolating activity of formaldehyde-treated VacA as determined by neutral red uptake assay of vacuolation induced in HeLa cells. Values are given as percentages of the activity of control VacA incubated under the same conditions as the samples but in the absence of formaldehyde. Results of a representative experiment are shown, with standard deviations calculated for triplicate samples. Values above the bars are 95% confidence limits.

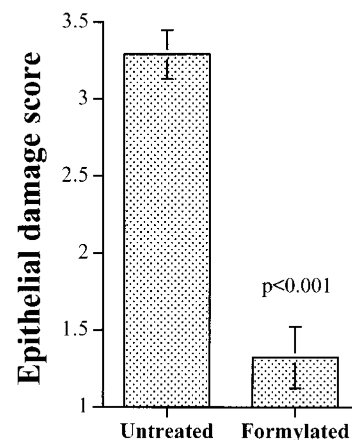

FIG. 2. In vivo toxicity of formaldehyde-treated VacA. Epithelial damage scores from histological analysis of gastric mucosae from mice treated with native VacA or VacA treated with 1.6 mM formaldehyde are shown. Error bars indicate the standard deviations of the mean damage observed in each group of mice.

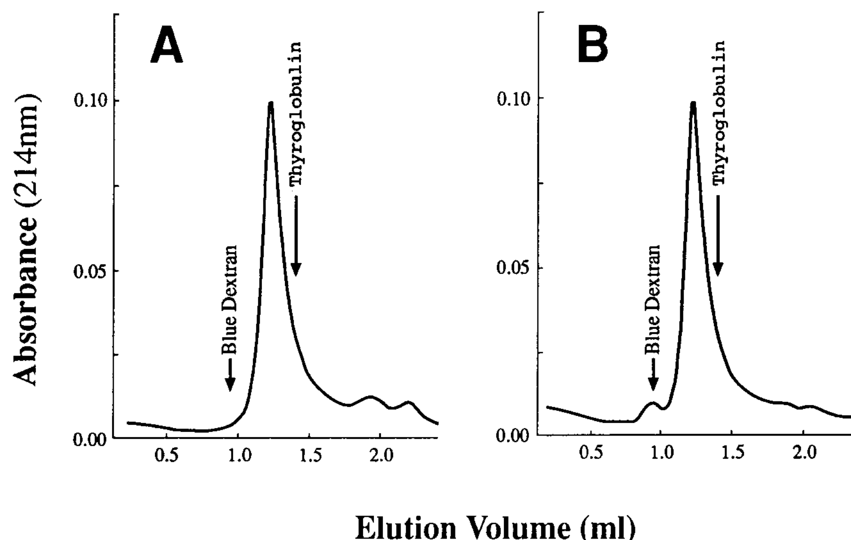

FIG. 3. Analytical gel filtration of formaldehyde-treated VacA. Results of Superose 6 chromatography in a SMART system of untreated VacA (A) or VacA after treatment with 3.2 mM formaldehyde (B) are shown. The elution volumes of molecular mass standards (blue dextran, 2,000 kDa; thyroglobulin, 669 kDa) used to calibrate the column are shown.

gel. Thus, while the excess free lysine prevented interoligomer cross-linking, the vicinity of the molecules in the oligomer permitted extensive intermonomer cross-linking at the higher concentrations of formaldehyde used.

The integrity of the mildly formylated VacA was further confirmed by direct visualization of individual molecules by electron microscopy. As shown in Fig. 5, after treatment with concentrations of formaldehyde which completely inactivated the toxin, the hexameric and heptameric structures of the molecules were still clearly visible. At the higher concentrations of formaldehyde used, the quality of the structures observed tended to deteriorate and an increase in the number of aggregates of several oligomers was observed.

Formaldehyde treatment has been used not only to inactivate toxic proteins but also to stabilize vaccine components against degradation (16). In fact, a genetically detoxified pertussis toxin, used in a commercially available vaccine, is nevertheless treated with formaldehyde to ensure stability during storage. After 7 days of incubation at 37°C, formaldehyde (1.6 mM)-treated VacA showed no reversion to toxicity in the HeLa cell assay and no significant difference from freshly treated toxin as determined by gel filtration, SDS-PAGE, or electron microscopy (data not shown).

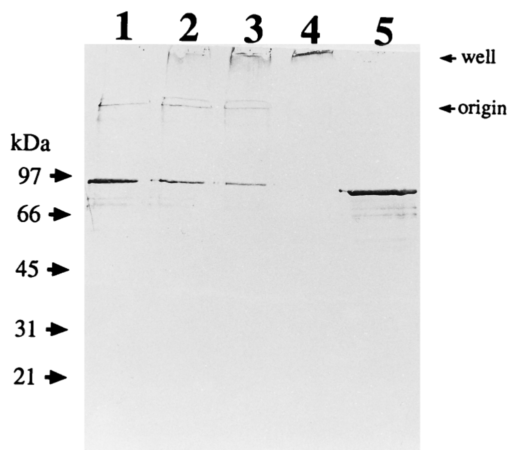

FIG. 4. Immunoblot of denaturing SDS-PAGE analysis of VacA after treatment with formaldehyde at 0.4 (lane 1), 0.8 (lane 2), 1.6 (lane 3), and 3.2 (lane 4) mM. The untreated toxin was also used (lane 5). The positions of molecular mass standards used in the electrophoresis (on the left), the junction between the stacking and running gels (origin), and the position of the loading wells (well) are indicated.

Garner and Cover (4) have shown by indirect immunofluorescence that native VacA and a 58-kDa carboxy-terminal proteolytic fragment bind to target cells. In a similar approach using immunofluorescence and flow cytometry, we show that after formaldehyde treatment the toxin is still capable of binding to HeLa cells (Fig. 6) but does not induce vacuolation. These data indicate that regions of the molecule involved in the interaction with target cells are not compromised by treatment with inactivating concentrations of formaldehyde.

Toxin-neutralizing epitopes have been found to be primarily conformational in that rabbit antisera raised against the native protein effectively inhibit toxic activity in the HeLa cell assay, whereas neutralization by antisera raised against the recombinant 95-kDa monomer polypeptide, which is unstructured and inactive, was undetectable (9). Furthermore, neutralizing antisera recognized primarily conformational epitopes, as measured in a comparative enzyme-linked immunosorbent assay using native and denatured antigens. Toxin treated with 1.6 mM formaldehyde induced antisera in rabbits with neutralizing titers only approximately 3.5-fold ($P < 0.01$) lower than those induced by untreated toxin (Fig. 7). Toxin treated with twice this concentration of formaldehyde also induced neutralizing antisera but at titers somewhat lower than those of the mildly treated or untreated proteins. Hence, the mildly formylated toxin, although completely inactive, retained a reasonable antigenic integrity.

To assess the potential of the toxoid as a component of a vaccine, mice were immunized intragastrically with the toxoid in combination with *E. coli* heat-labile enterotoxin as a mucosal adjuvant. In previous experiments, similar immunization with native toxin effectively protected mice from infection by mouse-adapted strains of *H. pylori* (10). Table 1 reports the levels of protection obtained with the formylated toxin com-

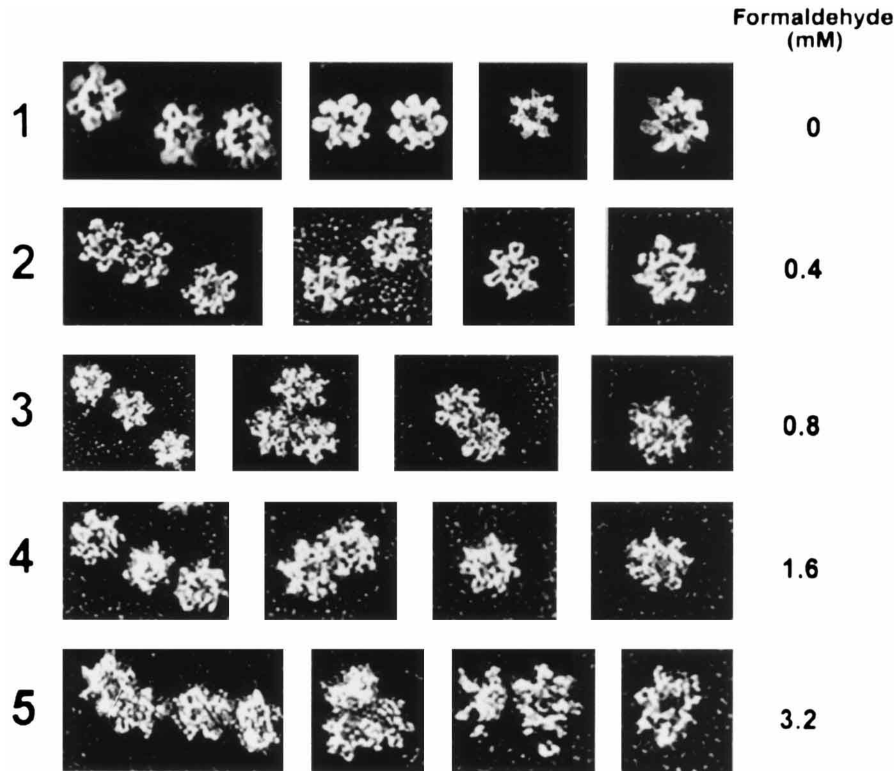

FIG. 5. Electron micrographs of formaldehyde-treated VacA prepared by quick-freeze deep etching. The concentration of formaldehyde used is shown at the right of each row of micrographs.

pared to those obtained with untreated toxin. No significant difference in levels of protection was found. Hence, the formaldehyde-inactivated toxin is an effective mucosal immunogen.

DISCUSSION

Toxin inactivation was achieved with very low concentrations of formaldehyde. In marked contrast, detoxification of pertussis toxin requires treatment with 250 mM formaldehyde for 1 week at 37°C (16). Even after this treatment, traces of toxic activity are still usually detected; however, pertussis toxin is extremely potent, and the in vitro assay is more sensitive than the assay for VacA. Nevertheless, VacA is clearly many times more sensitive to formaldehyde detoxification.

At minimal detoxifying concentrations of formaldehyde, cross-linking between the 95-kDa monomers of VacA was barely detectable, suggesting that inactivation was due to either

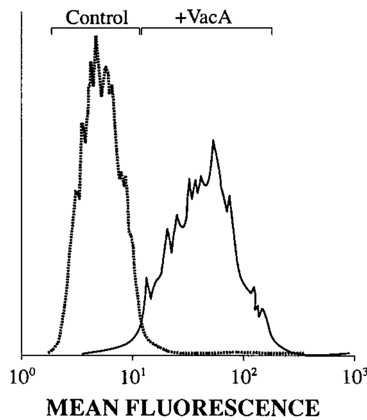

FIG. 6. Binding of formaldehyde (1.6 mM)-treated VacA to HeLa cells. Results of flow cytometric analysis of VacA bound to HeLa cells and revealed by using anti-VacA rabbit antiserum and fluorescein isothiocyanate-labelled anti-rabbit immunoglobulin are shown. Control cells were treated with both primary and secondary antibodies in the absence of VacA.

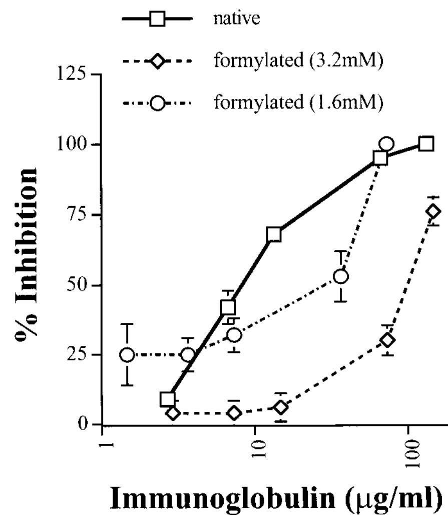

FIG. 7. Neutralization of VacA activity by hyperimmune rabbit sera raised against formaldehyde-treated VacA. Purified immunoglobulin from rabbit antisera raised against native or formaldehyde-treated VacA was incubated with active VacA in the HeLa cell vacuolation assay. Data are presented as percent inhibition of VacA activity versus antibody concentration. Error bars show the standard deviations of triplicate samples.

TABLE 1. Protection of mice from infectious challenge with *H. pylori* by oral administration of detoxified VacA

Treatment	No. of mice infected/total	% Protection
Saline	9/10	10
LT[a]	8/10	20
VacA	8/10	20
VacA + LT	2/10	80
f-VacA[b]	8/10	20
f-VacA + LT	3/10	70[c]

[a] LT, *E. coli* heat-labile toxin adjuvant.
[b] f-VacA, VacA detoxified with 1.6 mM formaldehyde.
[c] $P = 0.022$ versus the saline group and $P = 0.072$ versus the LT group as determined by Fisher's exact test for proportions.

cross-linking with free lysine molecules or linking between residues in physical proximity in the monomers. Notably, inactivation was achieved in the absence of extensive cross-linking between monomers. Taken together, these data indicate that 1 or 2 of the approximately 50 lysil residues in the VacA monomer are readily accessible to formaldehyde modification and are critical for activity.

After release from the bacteria, each 95-kDa monomer of VacA undergoes specific proteolytic cleavage to 37- and 58-kDa subunits which remain associated in the oligomeric structure (19). It was previously shown that mild treatment with formaldehyde results in fixing of the putative A and B subunits of native VacA to re-form a 95-kDa polypeptide which protects the oligomer from dissociation during preparation for electron microscopy (8). The cleavage between the two subunits in the VacA monomer occurs at a precise site in an 8-amino-acid repeat which is predicted to be flexible and surface exposed (19). This repeat contains four lysil residues. Hence, cross-linking at this site could conceivably block separation of the subunits and result in an inactive toxin. For other di-chain toxins, such as diphtheria toxin or pertussis toxin, the separation of the A subunit from the B subunit is essential for translocation of the A subunit across the membrane and hence for toxic activity. Formaldehyde detoxification of diphtheria toxin is associated with fixing together of the A and B subunits, and it has been suggested that this contributes to the inactivation of the molecule (13).

Alternatively, it may be that a lysil residue is involved in an active site of the VacA molecule. Amino groups, by virtue of their positive charge, are frequently found as functional parts of catalytic and allosteric sites. For example, arginine is an essential component of the active sites of several toxins with ADP-ribosylating activity (3), whereas lysil residues have been shown to be critical for the activity of RNase A and aspartate aminotransferase, among others (reviewed in reference 11). This is an attractive possibility which would indicate that it may be possible to genetically detoxify VacA by mutation of one or two lysines to uncharged residues.

In addition to being completely inactive in the HeLa cell assay, mildly formylated VacA was shown to be nontoxic upon intragastric administration to mice. Nevertheless, the detoxified protein retained the capacities to induce toxin-neutralizing antisera and to bind to target cells. Furthermore, oral administration of mildly formylated VacA was capable of conferring protective immunity against infectious challenge in a mouse model of *H. pylori* disease.

While several *H. pylori* antigens induce protective immunity to infection in mice (reviewed in reference 18), VacA is the only one which has been demonstrated to play a crucial role in gastric pathology. For this reason, detoxified VacA is an important candidate for inclusion in both prophylactic and therapeutic vaccines. The data presented here encourage the development of mildly formylated VacA for testing as an anti-*H. pylori* vaccine in human clinical trials.

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REFERENCES

1. **Cropley, I., G. Douce, M. Roberts, S. Chatfield, M. Pizza, I. Marsili, R. Rappuoli, and G. Dougan.** 1995. Mucosal and systemic immunogenicity of a recombinant, non-ADP-ribosylating pertussis toxin: effects of formaldehyde treatment. Vaccine **13:**1643–1648.
2. **De Bernard, M., E. Papinin, V. de Filippis, E. Gottardi, J. L. Telford, R. Manetti, A. Fontana, R. Rappuoli, and C. Montecucco.** 1995. Low pH activates the vacuolating toxin of Helicobacter pylori, which becomes acid and pepsin resistant. J. Biol. Chem. **270:**23937–23940.
3. **Domenighini, M., C. Montecucco, W. C. Ripka, and R. Rappuoli.** 1991. Computer modelling of the NAD binding site of ADP-ribosylating toxins: active-site structure and mechanism of NAD binding. Mol. Microbiol. **5:**23–31.
4. **Garner, J. A., and T. L. Cover.** 1996. Binding and internalization of the *Helicobacter pylori* vacuolating cytotoxin by epithelial cells. Infect. Immun. **64:**4197–4203.
5. **Ghiara, P., M. Marchetti, M. J. Blaser, M. K. R. Tummuru, T. L. Cover, E. D. Segal, L. S. Tompkins, and R. Rappuoli.** 1995. Role of the *Helicobacter pylori* virulence factors vacuolating cytotoxin, CagA, and urease in a mouse model of disease. Infect. Immun. **63:**4154–4160.
6. **Heuser, J. E.** 1989. Protocol for 3-D visualization of molecules on mica via the quick-freeze, deep etch technique. J. Electron Microsc. Tech. **13:**244–263.
7. **Jentoft, N., and D. G. Dearborn.** 1979. Labeling of proteins by reductive methylation using sodium cyanoborohydride. J. Biol. Chem. **254:**4359–4365.
8. **Lupetti, P., J. E. Heuser, R. Manetti, P. Massari, S. Lanzavecchia, P. L. Bellon, R. Dallai, R. Rappuoli, and J. L. Telford.** 1996. Oligomeric and subunit structure of the *Helicobacter pylori* vacuolating cytotoxin. J. Cell Biol. **133:**801–807.
9. **Manetti, R., P. Massari, D. Burroni, M. De Bernard, A. Marchini, R. Olivieri, E. Papini, C. Montecucco, R. Rappuoli, and J. L. Telford.** 1995. The *Helicobacter pylori* cytotoxin: importance of native conformation for induction of neutralizing antibodies. Infect. Immun. **63:**4476–4480.
10. **Marchetti, M., B. Aricò, D. Burroni, N. Figura, R. Rappuoli, and P. Ghiara.** 1995. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. Science **267:**1655–1658.
11. **Means, G. E., and R. E. Feeney.** 1995. Reductive alkylation of proteins. Anal. Biochem. **224:**1–16.
12. **Nencioni, L., G. Volpini, S. Peppoloni, M. Bugnoli, T. De Magistris, I. Marsili, and R. Rappuoli.** 1991. Properties of pertussis toxin mutant PT-9K/129G after formaldehyde treatment. Infect. Immun. **59:**625–630.
13. **Paliwal, R., and E. London.** 1996. Comparison of the conformation, hydrophobicity, and model membrane interactions of diphtheria toxin to those of formaldehyde-treated toxin (diphtheria toxoid): formaldehyde stabilization of the native conformation inhibits changes that allow membrane insertion. Biochemistry **35:**2374–2379.
14. **Papini, E., M. Bugnoli, M. De Bernard, N. Figura, R. Rappuoli, and C. Montecucco.** 1995. Bafylomycin A1 inhibits *Helicobacter pylori*-induced vacuolization of HeLa cells. Mol. Microbiol. **7:**323–327.
15. **Ramon, G.** 1924. Sur la toxine et sur l'anatoxine diphtheriques. Ann. Inst. Pasteur **38:**1–18.
16. **Rappuoli, R.** 1994. Toxin inactivation and antigen stabilization: two different uses of formaldehyde. Vaccine **12:**579–581.
17. **Rappuoli, R., M. Pizza, A. Podda, T. De Magistris, and L. Nencioni.** 1996. Towards third generation whooping cough vaccines. Trends Biotechnol. **9:**232–239.
18. **Telford, J. L., and P. Ghiara.** 1996. Prospects of a vaccine against *Helicobacter pylori*. Drugs **52:**799–804.
19. **Telford, J. L., P. Ghiara, M. Dellorco, M. Comanducci, D. Burroni, M. Bugnoli, M. F. Tecce, S. Censini, A. Covacci, Z. Y. Xiang, E. Papini, C. Montecucco, L. Parente, and R. Rappuoli.** 1994. Gene structure of the Helicobacter pylori cytotoxin and evidence of its key role in gastric disease. J. Exp. Med. **179:**1653–1658.

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