Brief Definitive Report

Selective Inhibition of Ii-dependent Antigen Presentation by *Helicobacter pylori* Toxin VacA

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Summary

A major virulence factor in the stomach chronic infection by *Helicobacter pylori* is a protein toxin (VacA), which alters cell membrane trafficking of late endosomal/prelysosomal compartments. Its role in the chronic infection established by *H. pylori* is unknown. To test the possibility that VacA alters antigen processing taking place in prelysosomal compartments, we have used the well-established model of antigen processing and presentation consisting of tetanus toxoid–specific human ($CD4^+$) T cells stimulated by autologous antigen-pulsed Epstein-Barr virus-transformed B cells. We found that VacA interferes with proteolytic processing of tetanus toxin and toxoid and specifically inhibits the Ii-dependent pathway of antigen presentation mediated by newly synthesized major histocompatibility complex (MHC) class II, while leaving unaffected the presentation pathway dependent on recycling MHC class II. The results presented here suggest that VacA may contribute to the persistence of *H. pylori* by interfering with protective immunity and that this toxin is a new useful tool in the study of the different pathways of antigen presentation.

More than 50% of the world population is infected with *Helicobacter pylori*, but most infections remain asymptomatic and only 10% of infected people become sick at some point in their life (1, 2). A close correlation has been established between the prolonged infection of the human stomach mucosa by *H. pylori* and the development of gastritis, and gastroduodenal ulcers, and with an increased risk of developing adenocarcinomas and other gastric tumors (1–3). In fact, *H. pylori* has been classified as a class I cancerogenic agent, being one of the factors involved in the development of stomach cancers. This bacterium enters the mucus layer covering the stomach epithelium and colonizes the human gastric mucosa: such infection may persist for decades. Bacterial factors necessary for colonization (for review see reference 1) are the flagella, which make this bacterium highly motile, adhesins, which strongly bind the saccharide moiety of glycoproteins and glycolipids, and a powerful urease, which buffers the acid stomach environment by releasing ammonia. Biopsies from patients affected by gastroduodenal ulcers almost invariably contain *H. pylori* strains harboring a pathogenicity island (4), characterized by the presence of the gene encoding for the 128-kD CagA protein, the major *H. pylori* antigen. Such strains also produce a 145-kD precursor that is processed and released in the culture medium as a 95-kD protein toxin (VacA), whose role in *H. pylori* infection is unknown (5).

VacA perturbs endocytosis at a prelysosomal stage in a process requiring the activity of the small GTPase Rab7 (6). This causes the formation and accumulation of compartments endowed with the vacuolar ATPase and with membrane markers both of late endosomes and lysosomes (6–8). In particular, the presence of Rab7 and lysosomal membrane glycoproteins, and the parallel absence of the cation-independent mannose 6-P receptor, allows the identification of those vesicles as an intermediate between late endosomes and lysosomes (7). A similar profile of markers is present in the compartments of APCs, where antigen proteolytic processing takes place (for review see reference 9).

Here, we have considered the possibility that VacA inhibits antigen processing by interfering with late endocytic membrane trafficking by APCs. This would in turn lower the proliferation of autologous human ($CD4^+$) T cells triggered by recognition of antigenic epitopes bound to MHC class II molecules exposed on APC surfaces (10). We have used the well-defined cellular system of antigen processing and presentation consisting of human tetanus toxoid (TT)–

M. Molinari and C. Galli wish to dedicate this work to the memory of Roland Graf.

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specific ($CD4^+$) T cells stimulated by autologous antigen-pulsed EBV-transformed B cells (10). TT is the most used human vaccine and its proteolytic processing and presentation by human lymphoid cells in culture has been intensively investigated (10–13). By using T cell clones with different specificity, we found that VacA interferes with the generation of T cell epitopes loaded on newly synthesized MHC class II molecules (the Ii-dependent pathway of antigen presentation), leaving unaffected generation and presentation of epitopes by class II molecules that recycle through early endosomal compartments (invariant chain [Ii]–independent pathway).

Materials and Methods

Cell Culture. EBV-B cell lines, clones Fc4m-, Fc7-, ANTEBV-, and KSEBV-B cells (donors A.L. and K.S.) were maintained at 37°C in a 5% CO_2 atmosphere in RPMI 1640, 2 mM l-glutamine, 1 mM Na-pyruvate, 50 μg/ml kanamycin, 50 μM 2-ME, and 1% nonessential amino acids (culture medium; GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS (Hyclone Laboratories, Inc., Logan, UT).

Intoxication of EBV-B Cells with VacA and Antigen Pulse. Cells were washed and resuspended to a density of 10^6 cells/ml in culture medium supplemented with 2% FCS. Highly purified toxin (VacA) of *H. pylori*, strain CCUG17874, was added. After 4 h of incubation at 37°C, cells were washed, resuspended (10^6 cells/ml) in culture medium supplemented with 10% FCS, and pulsed for an additional 4 h at 37°C with the antigens tetanus toxin (TeNT), recombinant human epidermal growth factor (EGF; Sigma Chemical Co., St. Louis, MO) and TT (Chiron Vaccines, Siena, Italy). Radioactive antigens ^{125}I-TeNT, labeled with Bolton-Hunter reagent (Amersham Internatioanl, Little Chalfont, UK), and ^{125}I-EGF, labeled with Iodo-Gen (Sigma Chemical Co.) were used for the experiments reported in Fig. 1 and Table 1.

VacA Inhibition of TT Processing in EBV-B Cells. Cells were incubated with 100 nM VacA and control cells were treated with boiled toxin or PBS. After a 1 h incubation at 4°C with ^{125}I-TeNT, cells were washed with ice-cold PBS/0.5% BSA and chased at 37°C in culture medium. The release of TCA-soluble radioactivity was measured with a γ counter (Multi-Prias; Packard, Meriden, CT).

Determination of ^{125}I-TeNT Degradation Pattern in TT-specific EBV-B Cells. TT-specific EBV-B cells (clone Fc4m) were pretreated with 100 nM VacA as described above, pulsed for 2 h at 4°C with the radioactive antigen, and then chased for 4 h at 37°C. Whole cell extracts were subjected to SDS-PAGE. The gel was dried under a vacuum and subjected to autoradiography. Autoradiograms were scanned with a dual wavelength Shimadzu CS 630 densitometer.

T Cell Proliferation Assays. T cell clones specific for TT-derived peptides (ALT15 [epitope P30], ALT81 [P7], ALT172 [P3], ALT210 [C-fragment], ALT220 [P30, from donor A.L.], and KSMix98 and KSMix140 [P2, from donor K.S.]) were stimulated with autologous EBV-B cells preincubated with 100 nM VacA and then pulsed with TT (see Fig. 2, *a–c*) or with the KSMix98 epitope P2, whose sequence is QYIKANSKFIGITE (see Fig. 2 *c*). Control EBV-B cells were mock treated. The overnight incorporation of [^{3}H]thymidine in T cells was determined after 48 h of proliferation.

NK Cells Cytotoxicity Assay. The effect of VacA on NK cells cytotoxicity was tested on NK1- and NK2-specific cell clones (14). NK cells were pretreated with 100 nM VacA or were mock treated. A mutant EBV-transformed B cell line (721.221) expressing no HLA class I molecules (15), which is susceptible both to NK1- and NK2-specific cell lines, was used as a target and loaded with ^{51}Cr during a 2 h incubation in a medium containing 100 μCi of $Na^{51}Cr/10^6$ cells. The cytotoxic activity of VacA-treated and control effector (E) NK cells was tested using 5,000 target (T) EBV-B cells and increasing numbers of NK cells.

Results and Discussion

Antigen Processing Is Defective in EBV-B Cells Exposed to VacA. Fig. 1 *a* shows that VacA alters the antigen processing compartment of APCs in such a way that the extent of proteolytic processing of ^{125}I-TeNT, as determined by release in the culture medium of TCA-soluble radioactivity, is significantly diminished. This holds true for EBV-B cells capturing externally added antigen by fluid phase (KS- and ANT-EBV-B cell clones) or by surface immunoglobulin-mediated endocytosis (TT-specific EBV-B cell clones Fc4m and Fc7; Table 1). Similarly, VacA inhibits the degradation of ^{125}I-EGF (Table 1), which is taken up by EBV-B cells lacking the EGF receptor (16) via fluid phase endocytosis. These results confirm and extend recent findings showing that VacA inhibits the degradation of receptor-mediated endocytosed EGF in HeLa cells (8), and they indicate that such an inhibition is a general consequence of VacA cell intoxication. The dose–response curve of Fig. 1 *a* shows that low nM toxin concentrations are inhibitory and that maximal inhibition (~60%) is reached at 250–500 nM. Significantly, VacA never completely blocks the degradation of endocytosed material, even at higher concentrations.

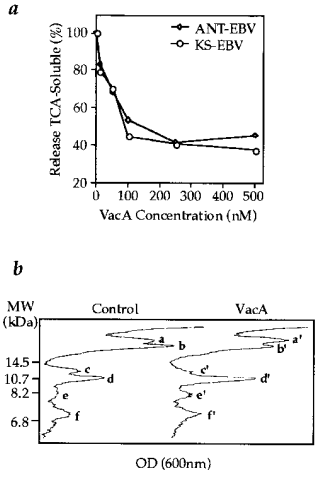

Figure 1. Effect of VacA on the proteolytic processing of ^{125}I-TeNT by EBV-B cell clones. (*a*) ^{125}I-TeNT–derived TCA-soluble radioactive peptides released in the culture medium by cells exposed to increasing VacA concentrations. After incubation with 250 nM VacA, the two APC clones release in the medium ~40% of the TCA-soluble radioactivity released by control cells. ANT– and KS–EBV-B internalize antigens via fluid phase endocytosis, but parallel experiments with clones Fc4m and Fc7, which internalize antigen via surface Ig, gave similar results (see Table 1). Values are representative of sets of at least three independent experiments. (*b*) *Left*, control cells; *right*, VacA-treated cells. The pattern of gel-associated radioactivity determined by densitometric scanning of an autoradiogram shows differences in ^{125}I-TeNT processing, after VacA-treatment of the TT-specific EBV-B cell clone Fc4m; the ratio between peaks *a* and *b* (control cells) changes upon treatment of the APCs with VacA (*a′* and *b′* in VacA-treated cells); fragment *c* is not formed in cells treated with the toxin, whereas formation of peaks *d′* and *e′* increases with respect to control cells.

Table 1. *Effect of VacA on Antigens Degradation by Different EBV-B Cell Clones*

EBV-B cell clone (antigen tested)	Release of TCA-soluble radioactivity (% of control)
ANT-EBV (^{125}I-TeNT)*	45 (±10)
ANT-EBV (^{125}I-EGF)*	40 (±10)
KS-EBV (^{125}I-TeNT)*	38 (±10)
KS-EBV (^{125}I-EGF)*	35 (±10)
Fc4m (^{125}I-TeNT)‡	40
Fc7 (^{125}I-TeNT)‡	40

The toxin shows comparable inhibitory effect on the degradation of antigens (^{125}I-TeNT and ^{125}I-EGF) endocytosed *via fluid phase (ANT– and KS-EBV-B cell clones) or ‡by surface immunoglobulin-mediated process (Fc4m and Fc7 clones).

Experiments aimed at identifying possible changes of the antigen degradation pattern upon toxin treatment gave the results presented in Fig. 1 *b*. Interestingly, the comparison of the densitometric analysis of the fragment-associated radioactivity of control (Fig. 1 *b*, *left*) versus VacA-treated cells (Fig. 1 *b*, *right*) indicates that not only the extent, but also the electrophoretic pattern, of antigen degradation changes upon treatment with the toxin (see figure legend). Recently, VacA was shown to increase the pH of intracellular acidic compartments of HeLa cells (8). In APCs, the acidic lumen of these compartments, and the highly reducing environment, are essential for antigen processing, since they promote antigen unfolding and maximize the activity of proteases that operate optimally at low pH values (9, 17). Although no reliable measurement of the pH of the antigen-processing compartment of EBV-B cells could be obtained, the strong analogies observed upon intoxication of epithelial cells and APCs (impaired degradation of endocytosed material, vacuolization, and lack of effect on protein synthesis) suggest that neutralization of the antigen-processing compartment by VacA could substantially contribute to the effects described here. Moreover, it has been clearly documented that neutralization of those compartments by lysosomotropic agents decreases the amount of processed antigen and changes its degradation pattern. Thus, the VacA-induced decrease in the degradation of antigens by APC can be mimicked by the use of compounds such as chloroquine, quinidine, or concanamycin B (10, 13, 18, and results not shown).

VacA Inhibits T Cell Proliferation Triggered by Ii-dependent Antigen Presentation. Since T cell response is based on the highly specific, MHC class II–restricted presentation of antigenic epitopes at the surface of APCs (19, 20), the effect of VacA on the amount (and the type) of TT epitopes produced may result in an indirect inhibition of the proliferation of CD4$^+$ T cells. In fact there is a direct correlation between the release of acid soluble antigen fragments by APCs and their ability to stimulate T cells (18).

MHC class II molecules present peptides derived from the endosomal–phagosomal system to CD4$^+$ T cells (9, 17, 21). MHC class II α/β heterodimers associate early during biosynthesis with the Ii, which assists the correct assembly of class II molecules, prevents premature endogenous peptide binding in the endoplasmic reticulum (21), and directs MHC class II to the antigen-processing compartment via a

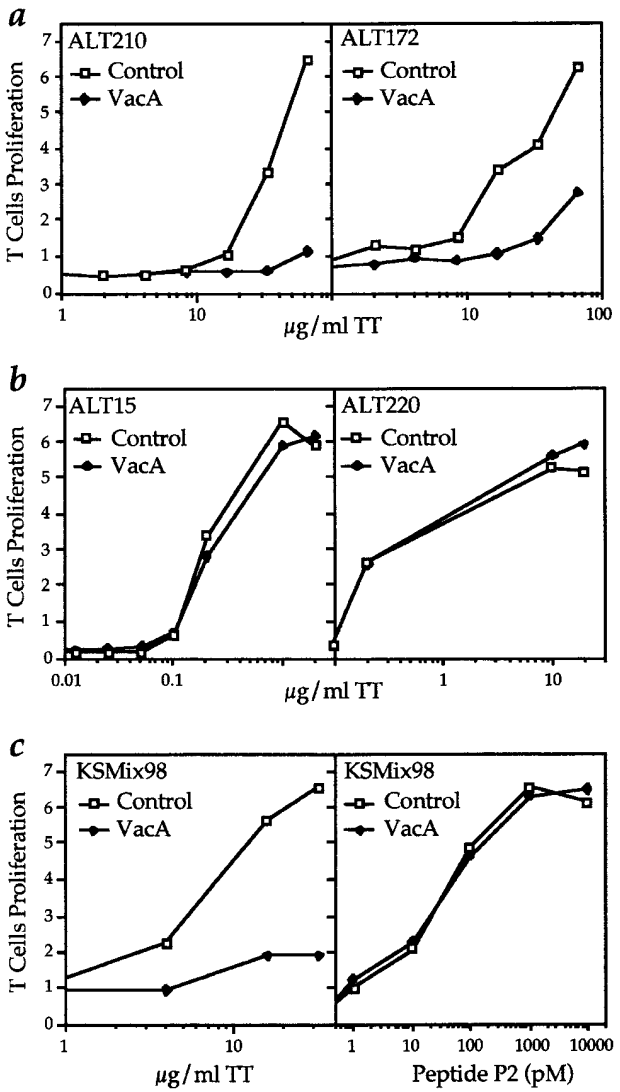

Figure 2. Effect of VacA on processing and presentation of different T cell epitopes. T cell clones specific for TT-derived peptides were stimulated with autologous EBV-B cells preincubated for 4 h with 100 nM VacA or mock-treated and then pulsed with TT. (*a*) Inhibition by VacA of the antigen presentation to T cell clones ALT210 and ALT172, which recognize epitopes that have been generated in late endocytic compartments and loaded onto newly synthesized MHC class II. Overlapping results were obtained with clones ALT81 and KSMix140 (data not shown) and with the clone KSMix98 as shown in the left of *c*. (*b*) Lack of effect of VacA on the B cell–induced proliferation of T cell clones ALT15 and ALT220, whose receptors recognize antigen–MHC class II complexes, which assemble in a recycling compartment independently from de novo protein synthesis. (*c*) *Left*, inhibition by VacA of the presentation to T cell clone KSMix98; *right*, the inhibition is circumvented by addition of the KSMix98-specific T cell epitope P2.

leucine-based motif present in its cytosolic tail (22). The Ii-dependent pathway of antigen presentation is inhibited by inhibitors of protein synthesis (23, 24) and by lysosomotropic agents that increase the lumenal pH of the endo-lysosomal compartments and thus prevent generation of epitopes (13, 18). However, in an alternative Ii-independent pathway of antigen presentation, other epitopes are generated in less acidic early endocytic compartments and are loaded on mature MHC class II molecules that recycle from the cell surface (25, 26) similarly to the transferrin receptor–transferrin complex. This route is less sensitive to lysosomotropic agents (25, 26) and is independent of protein synthesis. The two pathways complement each other and ensure maximal presentation of the several antigenic determinants present in most antigens. In the case of TT, it is possible to distinguish the two antigen presentation pathways because T cell clones with different specificity have been characterized. Some of them (ALT81, ALT172, ALT210, KSMix98, and KSMix140), recognize and are activated by epitopes generated in late endocytic compartments and loaded onto newly synthesized MHC class II molecules. Other clones (ALT15 and ALT220) recognize epitopes generated in early endocytic compartments and loaded onto recycling MHC class II molecules. Fig. 2, *a* and *c*, *left*) show that VacA pretreatment of APCs strongly inhibits the TT-dependent proliferation of T cell clones of the former type (ALT210, ALT172 and KSMix98), whereas the response of T cell clones of the latter type (ALT15 and ALT220) remains unaffected (Fig. 2 *b*). The finding that the Ii-independent antigen presentation via recycling MHC class II molecules is not affected is in keeping with the lack of VacA effect on transferrin recycling (8) and confirms that early stages of endocytosis, in this case the generation of epitopes in the mildly acidic early endosomes, are not affected by VacA. On the other hand, the toxin released strongly influences processes that occur in the distal part of the endocytic path and, in the case of APCs, heavily interferes with the extensive antigen degradation required for the generation of T epitopes to be loaded on newly synthesized MHC class II molecules.

VacA Does Not Affect MHC Class II Antigen Loading and Epitope Presentation to T Cells. Fig. 2 *c* shows that treatment of APCs with VacA strongly inhibits generation and presentation of the specific KSMix98 epitope (QYIKANSKFIGITE, peptide P2). However, proliferation of the KSMix98 clone is reestablished (*Fig. 2 c*, *right*) upon addition of the P2 peptide to VacA-treated APCs. This clearly indicates that VacA does not directly perturb the epitope–MHC class II complex, nor does it interfere with the interaction of epitope-loaded class II molecules with specific T cells. Additional experiments performed by FACS® analysis confirmed that VacA alters neither the total amount of MHC class II and I molecules nor the number of surface exposed class II and I molecules on EBV-B cells (data not shown). Such findings are in complete agreement with the fact that VacA does not interfere with protein synthesis and protein secretion (Papini, E., and Satin, B., unpublished data) and with the membrane traffic pathways connecting the endoplasmic reticulum to the cell surface (8). The selective inhibition of the Ii-dependent process of antigen presentation by VacA makes it a novel tool for distinguishing between the different pathways of antigen presentation.

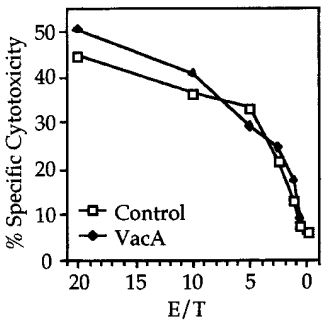

Figure 3. VacA does not influence NK cell–mediated cytotoxicity. Two NK cell clones (NK1 and NK2) were tested for their cytotoxic activity on a mutant EBV-transformed cell line not expressing HLA class I molecules. The radioactivity released in the culture medium after 4 h of incubation at different E/T ratios is plotted in the figure. The experiment reported is representative of a set of three different experiments.

VacA Does Not Inhibit NK Cell Cytotoxicity. Loading of T epitopes on newly synthesized MHC class II molecules is followed by migration and exocytosis of the complexes to the cell surface. In principle this can occur via different intracellular trafficking pathways, such as direct exocytosis of the antigen processing compartment, as suggested by recent evidence (27) or migration to the trans Golgi network followed by exocytosis or recycling to early endosomal compartments. However, knockout of early endosomes does not affect EBV-B cell presentation of TT epitopes to specific T cells (11) and VacA does not interfere with the movement of newly synthesized proteins from the endoplasmic reticulum to the surface in HeLa cells (8). Substantial similarities are apparent between surface expression of MHC class II molecules and the exocytosis of perforin containing granules of NK cells (27, 28). NK cells play a major role in the killing of MHC class I–negative tumor cells (29) and *H. pylori* prolonged infection increases the probability of development of stomach cancers. These considerations prompted us to investigate the effect of VacA on NK cell cytotoxicity. Fig. 3 shows that the toxin does not inhibit such a process. Together with previous evidence that VacA affects a restricted and late segment of the endocytic pathway (6), these results make it unlikely that VacA inhibits T cell proliferation by interfering with the movement of the antigen–MHC class II complex from the assembly compartment to the cell surface.

Conclusions. We have described here a novel activity of the VacA toxin released by pathogenic strains of *H. pylori*, which leads to an impairment of the stimulation of antigen specific $CD4^+$ T cells by APCs that present antigen through the Ii-dependent pathway. The data presented clearly shows that VacA depresses the stimulation of T cell proliferation induced by APCs by lowering the amount of T cell epitopes generated in the antigen-processing compartments. Since lysosomotropic agents were found to mimic the effect of VacA intoxication (inhibiting and partially changing the pattern of antigen degradation; reference 13), we postulate that these results can be explained accordingly. It is tempting to speculate that the differential effect of VacA on the different antigen presentation modes may lead to pref-

erential stimulation of T helper subpopulations (30). This study indicates that VacA may be used as a novel and powerful tool for discrimination between antigen presentation requiring the entry of the antigen in a late endosomal/prelysosomal compartment and antigen presentation not requiring this.

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