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+1) 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 carcinogenic 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)-
Materials and Methods

Cell Culture. EBV-B cell lines, clones Fc4m-, Fc7-, AN-TEBV-, and KSEBV-B cells (donors A.L. and K.S.) were maintained at 37°C in a 5% CO2 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, M D) supplemented with 10% FCS (Hyclone Laboratories, Inc., Logan, UT).

Toxin Treatment of EBV-B Cells with VaA and Antigen Pulse. Cells were washed and resuspended to a density of 106 cells/ml in culture medium supplemented with 2% FCS. Highly purified toxin (VaA) of H. pylori, strain CUG17874, was added. After 4 h of incubation at 37°C, cells were washed, resuspended (106 cells/ml) in culture medium supplemented with 10% FCS, and pulsed for an additional 4 h at 37°C with the antigen tetanus toxin (TeNT), recombinant human epidermal growth factor (EGF; Sigma Chemical Co., St. Louis, M O) and TeNT (Chiron Vaccines, Siena, Italy). Radioactive antigens [125I]-TeNT, labeled with Bolton-Hunter reagent (Amersham International, Little Chalfont, UK), and [125I]-EGF, labeled with Iodo-Gen (Sigma Chemical Co.) were used for the experiments reported in Fig. 1 and Table 1.

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

Determination of [125I]-TeNT Degradation Pattern in TT-specific EBV-B Cells. TT-specific EBV-B cells (clone Fc4m) were pretreated with 100 nM VaA 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.

Toxin Treatment of NK Cells. 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 KSM1x98 and KSM1x140 [P2, from donor K.S.]) were stimulated with autologous EBV-B cells preincubated with 100 nM VaA and then pulsed with TT (see Fig. 2, a–d) or with the KSM1x98 epitope P2, whose sequence is QYIKANSKFIGITE (see Fig. 2 d). Control EBV-B cells were mock treated. The overnight incorporation of [3H]thymidine in T cells was determined after 48 h of proliferation.

NK Cells Cytotoxicity Assay. The effect of VaA on NK cells cytotoxicity was tested on NK1- and NK2-specific cell clones (14). NK cells were pretreated with 100 nM VaA 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 51Cr during a 2 h incubation in a medium containing 100 μCl of Na251Cr/106 cells. The cytotoxic activity of VaA-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 Effective in EBV-B Cells Exposed to VaA. Fig. 1 a shows that VaA alters the antigen processing compartment of APCs in such a way that the extent of proteolytic processing of [125I]-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 AN-TEBV-B cell clones) or by surface immunoglobulin-mediated endocytosis (TT-specific EBV-B cell clones Fc4m and Fc7; Table 1). Similarly, VaA inhibits the degradation of [125I]-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 VaA inhibits the degradation of receptor-mediated endocytosed EGF in HeLa cells (8), and they indicate that such an inhibition is a general consequence of VaA 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, VaA never completely blocks the degradation of endocytosed material, even at higher concentrations.

Figure 1. Effect of VaA on the proteolytic processing of [125I]-TeNT by EBV-B cell clones. (a) [125I]-TeNT-derived TCA-soluble radioactive peptides released in the culture medium by cells exposed to increasing VaA concentrations. After incubation with 250 nM VaA, 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, VaA-treated cells. The pattern of gel-associated radioactivity determined by densitometric scanning of an autoradiogram shows differences in [125I]-TeNT processing, after VaA-treatment of the TT-specific EBV-B cell clone Fc4m, the ratio between peaks a and b (control cell) changes upon treatment of the APCs with VaA (a’ and b’ in VaA-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.

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Experiments aimed at identifying possible changes of the antigen degradation pattern upon toxin treatment gave the results presented in Fig. 1. Interestingly, the comparison of the densitometric analysis of the fragment-associated radioactivity of control (Fig. 1b, left) versus VacA-treated cells (Fig. 1b, 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).

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 (125I-TeNT)*                    | 45 (+10)                                          |
| ANT-EBV (125I-EGF)*                     | 40 (+10)                                          |
| KS-EBV (125I-TeNT)*                     | 38 (+10)                                          |
| KS-EBV (125I-EGF)*                      | 35 (+10)                                          |
| Fc4m (125I-TeNT)‡                       | 40                                                 |
| Fc7 (125I-TeNT)‡                        | 40                                                 |

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

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 T cell 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

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Figure 2. Effect of VacA on processing and presentation of different T cell epitopes. T cell clones specific for T cell-derived peptides were stimulated with autologous EBV-B cells pretreated 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 ALT15 and ALT220, whose receptors recognize antigen-MHC class II complexes, which assemble in a recycling compartment independently from de novo protein synthesis. (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 li-
dependent pathway of antigen presentation is inhibited by
inhibitors of protein synthesis (23, 24) and by lysosomotro-
opic agents that increase the lumenal pH of the endo-lysoso-
mal compartments and thus prevent generation of epitopes
(13, 18). However, in an alternative li-independent path-
way of antigen presentation, other epitopes are generated
in less acidic early endocytic compartments and are loaded
onto 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 lysoso-
motropic agents (25, 26) and is independent of protein
synthesis. The two pathways complement each other and
ensure maximal presentation of the several antigenic deter-
minants present in most antigens. In the case of TT, it is
possible to distinguish the two antigen presentation path-
ways because T cell clones with different specificity have
been characterized. Some of them (ALT81, ALT172,
ALT210, KSMix98, and KSMix140), recognize and are ac-
tivated by epitopes generated in late endocytic compart-
ments 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 li-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 epi-
topes 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 en-
docytic path and, in the case of APCs, heavily interferes with
the extensive antigen degradation required for the genera-
tion 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 KSM ix98 epitope (QYIKAN-
SKFQITGE, peptide P2). However, proliferation of the
KSM ix98 clone is reestablished (Fig. 2 c right) upon addition
of the P2 peptide to VacA-treated APCs. This clearly indi-
cates that VacA does not directly perturb the epitope-
MHC class II complex, nor does it interfere with the inter-
action 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 M HC
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 li-dependent process of antigen presentation by VacA
makes it a novel tool for distinguishing between the different
 pathways of antigen presentation.

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 in-
tracellular trafficking pathways, such as direct exocytosis
of the antigen processing compartment, as suggested by recent
evidence (27) or migration to the trans Golgi network fol-
lowed by exocytosis or recycling to early endosomal com-
partments. However, knock-out 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 move-
ment of newly synthesized proteins from the endoplasmic
reticulum to the surface in HeLa cells (8). Substantial simi-
larities 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 cancer. 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. To-
gether 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 sur-
f "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 mole-
cules. 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 rep-
resentative of a set of three dif-
ferent experiments.

Figure 3.
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