Coordinate Involvement of Invasin and Yop Proteins in a *Yersinia pseudotuberculosis* -Specific Class I-Restricted Cytotoxic T Cell-Mediated Response

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Coordinate Involvement of Invasin and Yop Proteins in a *Yersinia pseudotuberculosis*-Specific Class I-Restricted Cytotoxic T Cell-Mediated Response

Géraldine Falgarone,* Hervé S. Blanchard,* François Virecoulon,* Michel Simonet,‡ and Maxime Breban²*†

*Yersinia pseudotuberculosis* is a pathogenic enteric bacteria that evades host cellular immune response and resides extracellularly in vivo. Nevertheless, an important contribution of T cells to defense against *Yersinia* has been previously established. In this study we demonstrate that Lewis rats infected with virulent strains of *Y. pseudotuberculosis*, mount a *Yersinia*-specific, RT1-A-restricted, CD8⁺ T cell-mediated, cytotoxic response. Sensitization of lymphoblast target cells for cytolysis by *Yersinia*-specific CTLs required their incubation with live *Yersinia* and was independent of endocytosis. Although fully virulent *Yersinia* did not invade those cells, they attached to their surface. In contrast, invasin-deficient strain failed to bind to blast targets or to sensitize them for cytolysis. Furthermore, an intact virulence plasmid was an absolute requirement for *Yersinia* to sensitize blast targets for cytolysis. Using a series of *Y. pseudotuberculosis* mutants selectively deficient in virulence plasmid-encoded proteins, we found no evidence for a specific role played by YadA, YopH, YpkA, or YopJ in the sensitization process of blast targets. In contrast, mutations suppressing YopB, YopD, or YopE expression abolished the capacity of *Yersinia* to sensitize blast targets. These results are consistent with a model in which extracellular *Yersinia* bound to lymphoblast targets via invasin translocate inside eukaryotic cytosol YopE, which is presented in a class I-restricted fashion to CD8⁺ cytotoxic T cells. This system could represent a more general mechanism by which bacteria harboring a host cell contact-dependent or type III secretion apparatus trigger a class I-restricted CD8⁺ T cell response. The Journal of Immunology, 1999, 162: 2875–2883.

Three species of the Gram-negative bacteria genus *Yersinia* are pathogenic for humans and rodents. The most harmful, *Y. pestis*, is the causative agent of plague, whereas the closely related *Y. pseudotuberculosis* and the more distant *Y. enterocolitica* are responsible for gastrointestinal infections, manifested by acute ileitis and mesenteric lymphadenitis (1). Infection with the latter species is sometimes complicated with reactive sterile arthritis, especially in patients bearing the HLA-B27 class I MHC allele (2). Such manifestation is thought to be mediated by the immune system and may involve a pathogenic HLA-B27-restricted *Yersinia*-specific CD8⁺ T cell (3).

Enteric infections with *Y. pseudotuberculosis* and *Y. enterocolitica* share similar characteristics. After crossing the acidic environment of the stomach, bacteria reach the intestine and invade the lamina propria of the terminal ileum through M cells. The bacteria multiply in Peyrer’s patches and drain into the mesenteric lymph nodes (LN),³ causing acute mesenteric lymphadenitis. The chromosomal outer membrane protein invasin, which promotes binding to the β1 chain of integrins and internalization into eukaryotic cells in vitro (4), is necessary for efficient translocation of bacteria across the intestinal epithelium (5, 6). All pathogenic strains also harbor a 70- to 75-kb plasmid pYV that encodes several factors essential for bacterial virulence, e.g., Yops (*Yersinia* outer membrane proteins) and YadA (*Yersinia* adhesin). Upon contact of *Yersinia* with host target cell, Yops are secreted via a type III secretion system (7). The Yops fall into two major groups of proteins: YopB and YopD, which form a delivery apparatus, and translocate several other effector proteins (YopE, YopH, YpkA/ YopO, and YopM) directly into the eukaryotic cytosol through the plasma membrane (8). Although the function of all effector Yops has not been elucidated, some of them display antiphagocytic (YopH and YopE) and cytotoxic (YopE) activities or are involved in inducing macrophage apoptosis (YopJ), all activities that are probably implicated in the extracellular survival of *yersinia* and contribute to delay the development of a cell-mediated immune response (9–11).

It is established that T cells play an essential role in defense against *Yersinia* in mice (12). The protective role of IFN-γ-producing CD4⁺ T cells has been demonstrated (13, 14). Likewise, CD8⁺ lymphocytes were shown to mediate protection in adoptive transfer experiments (13). However, the specificity and function of those protective CD8⁺ cells have not been clearly defined. In particular, there was no demonstration that *Yersinia* infection elicits a class I-restricted specific CTL response in vivo, although such a possibility was suggested using an indirect experimental approach (15). Cytotoxic CD8⁺ T cells that recognize *Yersinia*-derived Ag presented in a class I-restricted fashion have been obtained from the joints of patients suffering from reactive arthritis. However, direct evidence that those T cells were actually generated in response to *Yersinia* infection is lacking (16, 17). One major issue with the presentation of Ag derived from an extracellularly located

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3 Abbreviations used in this paper: LN, lymph node; LEW, Lewis; LB, Luria broth; CCD, cytochalasin D; BMDM, bone marrow-derived macrophage.
bacterium by class I molecules is that such Ag presumably need to penetrate the cell to gain access to class I presentation pathway (18). To date, class I presentation has been shown to work efficiently for a limited number of bacteria that survive intracellularly, such as Listeria, Mycobacteria, Salmonella, and Chlamydia (19, 20).

In the present work we provide evidence that infection of rat with Y. pseudotuberculosis, an extracellularly living bacteria, elicits a bacteria-specific, class I-restricted, cytotoxic CD8 \(^+\) T cell response. The roles played by invasion and virulence plasmid-encoded proteins in this response are further characterized, and its functional implications are discussed.

**Materials and Methods**

**Rats**

Inbred Lewis (LEW), and Dark Agouti females, 2–3 mo old, were purchased from Iffa Credo (L’Arbresle, France) and housed under conventional conditions. Study procedures were approved by the institutional animal care committee.

**Bacterial strains and growth conditions**

*Y. pseudotuberculosis* strains (Table I) used in this study were grown in Luria broth (LB) medium, sometimes supplemented with 20 mM sodium oxalate/20 mM MgCl\(_2\) (Ca\(^{2+}\)-deficient LB medium) at 28 or 37°C. Strains of *Escherichia coli* 25922 obtained from American Type Culture Collection (Manassas, VA) and *Salmonella typhimurium* (patient isolate) were grown in LB medium at 37°C. The actual number of bacteria inoculum was determined by plating serial dilutions of the inoculum on LB agar and counting CFU after an incubation period of 48 h at 28 or 37°C.

**Tissue culture medium, mAbs, and other reagents**

Tissue cultures were performed in RPMI 1640 medium with Glutamax I (Life Technologies, Eragny-sur-Oise, France) supplemented with 5% FCS, penicillin G (100 U/ml), streptomycin (100 \(\mu\)g/ml), 0.02 mM 2-ME, and 5 mM HEPES unless otherwise stated. Murine anti-rat mAbs used are listed in Table II. The anti-*Yersinia* invasion mAb 3A2 (murine IgG2a) (39) was a gift from R. R. Isberg (Tufts University, Boston, MA). Irrelevant murine mAbs were \(\gamma\)C11 (anti-human CD82, IgG1; a gift from H. Conjeaud, Paris, France) and OKT3 (anti-human CD3, IgG2a; Cilag, Boulogne, France). Goat anti-mouse IgG-FITC was purchased from Eurobio (Les Ulis, France). Cytochalasin D (CCD) was obtained from Sigma (St. Quentin Fallavier, France).

**Infection of animals**

*Y. pseudotuberculosis* grown overnight at 28°C in Ca\(^{2+}\)-deficient LB medium were centrifuged and resuspended in sterile PBS. For intragastric infection, rats were given 10\(^9\) bacteria in 0.5 ml of PBS through a gastric tube for 3 consecutive days. For i.p. infection, rats received a single inoculum of 10\(^9\) bacteria in 0.5 ml of PBS.

**Generation of anti-Yersinia CTLs**

Peripheral and mesenteric LN cells harvested from infected rats were resuspended in culture medium containing 10% Con A-stimulated rat spleen and LN cell supernatant and 50 mM α-methyl-d-mannoside and restimulated for 4–5 days in 96-well U-bottom culture dishes (10\(^5\) cells/well) with *Y. pseudotuberculosis* as follows. Bacteria grown overnight at 28°C in LB medium followed by 4-h incubation at 37°C in Ca\(^{2+}\)-deficient LB medium to induce expression of virulence plasmid proteins were washed and resuspended in tissue culture medium without antibiotics and incubated with stimulator cells at a ratio of 10-90 bacteria/cell for 2 h at 37°C. Thereafter, infected stimulator cells were treated for 1 h at 37°C with gentamicin (100 \(\mu\)g/ml), washed, and irradiated (3000 rad) before use.

**Anti-H-Y CTLs**

CTLs specific for the rat male H-Y Ag were generated as previously described (40). Briefly, LEW females were primed in vivo by s.c. and i.p. injection of 2 \(\times\) 10\(^7\) LEW male pooled LN and spleen cells. LN cells harvested from LEW female primed >3 wk previously were resuspended in culture medium containing 10% Con A supernatant and 50 mM α-methyl-d-mannoside and restimulated for 5 days in 96-well U-bottom culture dishes (10\(^5\) cells/well) with irradiated (3000 rad) LN cells from LEW male (3 \(\times\) 10\(^5\) cells/well).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Characteristic</th>
<th>Source or Ref.</th>
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<tbody>
<tr>
<td>IP277</td>
<td>Wild type, pYV(^+)</td>
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</tr>
<tr>
<td>IP277c</td>
<td>IP277 derivative, pYV-cured</td>
<td>5</td>
</tr>
<tr>
<td>YPIII</td>
<td>Wild type, pYV(^+) (pIB1)</td>
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</tr>
<tr>
<td>YPIIIc</td>
<td>YPIII derivative, pYV-cured</td>
<td>21</td>
</tr>
<tr>
<td>YP212</td>
<td>YPII derivative, inv mutant</td>
<td>22</td>
</tr>
<tr>
<td>YP15</td>
<td>YPIII derivative, yadA mutant</td>
<td>23</td>
</tr>
<tr>
<td>YP15</td>
<td>YPIII derivative, yopH mutant</td>
<td>J. B. Bliska (unpublished)</td>
</tr>
<tr>
<td>YP15</td>
<td>YPIII derivative, yopE(_{27,29,210}) mutant</td>
<td>24</td>
</tr>
<tr>
<td>YP15</td>
<td>YPIII derivative, yopE mutant</td>
<td>25</td>
</tr>
<tr>
<td>YP15</td>
<td>YPIII derivative, yopJ mutant</td>
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</tr>
<tr>
<td>YP15</td>
<td>YPIII derivative, yopB yopD mutant</td>
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<td>YPIII derivative, yopB yopD mutant</td>
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<tr>
<td>YP15</td>
<td>YPIII derivative, yopD mutant</td>
<td>28</td>
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Table II. Murine anti-rat Ag mAbs used

<table>
<thead>
<tr>
<th>mAb</th>
<th>Isotype</th>
<th>Ag</th>
<th>Cell Population Marked</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>R73</td>
<td>IgG1</td>
<td>αβ TCR</td>
<td>All αβ T cells</td>
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</tr>
<tr>
<td>OX34</td>
<td>IgG2a</td>
<td>CD2</td>
<td>All rat T cells</td>
<td>30</td>
</tr>
<tr>
<td>OX8</td>
<td>IgG1</td>
<td>CD8 α-chain</td>
<td>T cell, NK cells</td>
<td>31</td>
</tr>
<tr>
<td>OX35</td>
<td>IgG2a</td>
<td>CD4</td>
<td>T cells, macrophages</td>
<td>32</td>
</tr>
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<td>IgG1</td>
<td>CD45 epitope</td>
<td>All B cells</td>
<td>33</td>
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<tr>
<td>OX18</td>
<td>IgG1</td>
<td>RT1 class I (several loci)</td>
<td>MHC class I(^+) cells</td>
<td>34</td>
</tr>
<tr>
<td>F16-4-11</td>
<td>IgG1</td>
<td>RT1-A</td>
<td>MHC class I(^+) cells</td>
<td>35</td>
</tr>
<tr>
<td>OX6</td>
<td>IgG1</td>
<td>RT1-B</td>
<td>MHC class II(^+) cells</td>
<td>36</td>
</tr>
<tr>
<td>OX17</td>
<td>IgG1</td>
<td>RT1-D</td>
<td>MHC class II(^+) cells</td>
<td>34</td>
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<tr>
<td>OX42</td>
<td>IgG2a</td>
<td>C3b receptor</td>
<td>Macrophages</td>
<td>37</td>
</tr>
<tr>
<td>3.2.3</td>
<td>IgG1</td>
<td>NKP-P1</td>
<td>All NK cells</td>
<td>38</td>
</tr>
</tbody>
</table>
90% FCS and 10% DMSO were thawed, resuspended at 2.5 × 10^6 cells/ml, and the cells were incubated for 3 h at room temperature (Con A blasts) or (DuPont-New England Nuclear, Boston, MA) was added to the cell pellet, at 20% supernatant from L929 cells as a source of macrophage CSF, and incubated. After incubation for 2 h at 37°C, nonadherent cells were removed, replated in medium without FCS and plated in bacteriological grade culture dishes. Medium without effectors (100 μl) or with 1 N HCl (100 μl) was dispensed in triplicate into 96-well U-bottom culture dishes. Medium without effectors (100 μl) or with 1 N HCl (100 μl) was also added to triplicate wells of targets to determine spontaneous and maximal lysis, respectively. Effectors and targets in the plates were centrifuged for 2 min, and the plates were incubated for 4 h at 37°C and then recentrifuged in the same manner. Supernatant was harvested from each well (50 μl) and counted in a 1450 Microbeta counter (Wallac, Turku, Finland). The percent specific lysis was computed by the formula:

\[ \text{Percent Specific Lysis} = \frac{\text{Spontaneous Release} - \text{Spontaneous Release}}{\text{Maximal Release} - \text{Spontaneous Release}} \times 100 \]

For cytotoxicity assays, restimulated LN cells harvested after 5 days of culture were washed and resuspended at 5 × 10^6 cells/ml in culture medium containing 10% FCS. Labeled target cells were diluted in the same medium to 5 × 10^4 cells/ml. Threefold dilutions of effectors (100 μl) and targets (100 μl) were dispensed in triplicate into 96-well U-bottom culture dishes. Medium without effectors (100 μl) or with 1 N HCl (100 μl) was also added to triplicate wells of targets to determine spontaneous and maximal lysis, respectively. Effectors and targets in the plates were centrifuged at 65 × g for 2 min, and the plates were incubated for 4 h at 37°C and then recentrifuged in the same manner. Supernatant was harvested from each well (50 μl) and counted in a 1450 Microbeta counter (Wallac, Turku, Finland). The percent specific lysis was computed by the formula:

\[ \text{Percent Specific Lysis} = \frac{\text{Spontaneous Release} - \text{Spontaneous Release}}{\text{Maximal Release} - \text{Spontaneous Release}} \times 100 \]

Cell-mediated cytotoxicity assay

Rat LN cell blast targets were generated by culture for 40 h with Con A as previously described (40). In some experiments Con A-stimulated blasts that had been kept frozen at −80°C or in liquid nitrogen at 10^7 cells/ml in 90% FCS and 10% DMSO were thawed, resuspended at 2.5 × 10^6 cells/ml in the same medium used for restimulation, and incubated for 18 h before being used. Bone marrow-derived macrophage (BMDM) targets were obtained as previously described (41). Briefly, bone marrow cells harvested from rat long bones were resuspended at 10^7 cells/ml in tissue culture medium without FCS. Labeled target cells were diluted in the same medium to 5 × 10^4 cells/ml. Threefold dilutions of effectors (100 μl) and targets (100 μl) were dispensed in triplicate into 96-well U-bottom culture dishes. Medium without effectors (100 μl) or with 1 N HCl (100 μl) was also added to triplicate wells of targets to determine spontaneous and maximal lysis, respectively. Effectors and targets in the plates were centrifuged at 65 × g for 2 min, and the plates were incubated for 4 h at 37°C and then recentrifuged in the same manner. Supernatant was harvested from each well (50 μl) and counted in a 1450 Microbeta counter (Wallac, Turku, Finland). The percent specific lysis was computed by the formula:

\[ \text{Percent Specific Lysis} = \frac{\text{Spontaneous Release} - \text{Spontaneous Release}}{\text{Maximal Release} - \text{Spontaneous Release}} \times 100 \]

Flow cytometric analysis of cell surface Ags

All procedures were conducted at 4°C in PBS/2% FCS/0.01% NaN₃. Cells (5 × 10^6) were incubated with saturating concentrations of the appropriate mAb for 30 min, washed, then incubated with FITC-conjugated monoclonal goat anti-mouse IgG for 30 min. After washing, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

In vitro magnetic cell depletions

Selected subsets of T cells were obtained after in vitro restimulation by negative selection using the following combinations of mouse mAbs, OX6, OX17, OX33, OX42, and 3.2.3, to purify all T cells plus either OX8 or OX35 to...
purify CD4+ or CD8+ T cells, respectively. All procedures were conducted at 4°C in PBS/4% FCS as follows. Cells were incubated with saturating concentrations of mAb for 15 min, washed, and further incubated with goat antimouse IgG-conjugated microbeads (Dynabeads M-450, Dynal, Oslo, Norway) at a ratio of 20 microbeads/cell. Cells were washed again, resuspended in PBS-FCS, and sorted with a magnet (Dynal MPC).

**Ab inhibition assay**

Target cells were preincubated with dilutions of mAb at 4°C for 15 min in assay medium and then combined in 96-well plates as described above at an E:T cell ratio of 33. Triplicate wells were also plated containing target cells incubated with Ab at each concentration but lacking effectors. Results were expressed as the percent inhibition = \[1 - \left(\frac{\text{% lysis with mAb}}{\text{% lysis without mAb}}\right)\] × 100.

**Invasion assays**

For invasion assays, 10^6 blast target cells were incubated in 1 ml of culture medium without antibiotics in 24-well culture plates with live *Yersinia* at a ratio of 50 bacteria/cell in the same conditions as those used for the cell-mediated cytotoxicity assay (i.e., 2 h at 37°C followed by 1 h at 37°C with gentamicin) and washed to eliminate gentamicin. Internalized bacteria were released from infected cells by adding 1% Triton X-100 (Sigma) and measuring viable counts on agar plates. The entry of *Y. pseudotuberculosis* was expressed as the percentage of intracellular bacteria = (number of bacteria surviving gentamicin treatment/number of input bacteria) × 100.

**Light microscopy of cytospin smears**

To prepare cytospins, blast cells were incubated with live bacteria at a ratio of 50 bacteria/cell for 1 h at 37°C followed by 1 h at 37°C with gentamicin and were spun down at 72.3 × g for 8 min in a Cytospin III (Shandon, Eragny sur Oise, France). Bacteria and lymphoblasts were examined under light microscope after May-Grünwald-Giemsa staining.

**Results**

**Rats infected with *Y. pseudotuberculosis* mount a bacteria-specific CTL response**

To determine whether a CTL response is primed during infection with *Y. pseudotuberculosis*, LEW rats were infected intragastrically or i.p. with strains of *Y. pseudotuberculosis* harboring its virulence plasmid pYV. Rats were killed from 1–8 wk after infection, and LN cells were restimulated on irradiated *Y. pseudotuberculosis*-infected LN cells as a source of APCs. As shown, CTLs were generated after intragastric infection that efficiently killed LN blasts or BMDM targets infected in vitro with wild-type (pYV+), but not plasmid-cured (pYV−) *Y. pseudotuberculosis* (Fig. 1, A and B). Infection with either strain YPIII (Fig. 1A) or strain IP2777 (Fig. 1B) could efficiently prime rats for this specific CTL response. Furthermore, infection with *Y. pseudotuberculosis* strain IP2777 cross-primed the CTL response against the YPIII strain (Fig. 1B). In kinetics studies the CTL response peaked between 2 and 4 wk after infection (not shown). Likewise, a CTL response was generated in rats infected i.p. (not shown).

In vivo infection with *Y. pseudotuberculosis* was required to prime rat LN cells for this anti-*Yersinia* CTL response. Indeed, LN cells from uninfected rats that were restimulated in vitro on *Y. pseudotuberculosis*-infected LN cells failed to lyse *Y. pseudotuberculosis*-infected blast targets (not shown). Furthermore, following intragastric infection a significant CTL response was detectable in mesenteric LN cells but not in peripheral LN cells, indicating specificity of priming for LN cells afferent to the bacterial entry site (Fig. 1C). Finally, CTLs primed against the male H-Y minor histocompatibility Ag failed to lyse female targets infected with virulent *Y. pseudotuberculosis* (Fig. 1D).

This anti-*Yersinia* CTL response was specific for syngeneic cells infected with *Y. pseudotuberculosis*, since neither targets from another inbred strain of rats (Fig. 2A) nor targets infected with unrelated bacteria, such as *E. coli* or *S. typhimurium*, were efficiently killed (Fig. 2B).
examined by culturing bacteria released from blast cells incubated with *Yersinia*. These experiments showed the lack of invasion of rat lymphoblasts by live *Yersinia* (Table III). By contrast, flow cytometry using an anti-invasin mAb demonstrated the presence of *Yersinia* at the surface of >40% of blast targets (Fig. 5), readily detectable upon contact of intact *Yersinia* with target cells, suggesting that interaction of live *Yersinia* with the target cell surface was necessary to sensitize targets for killing and occurred via attachment of the bacteria to the cell membrane. In addition, experiments using CCD indicated that sensitization of blast targets for lysis was independent of endocytosis (Fig. 4). An intact Yop delivery apparatus and YopE are both required to sensitize blast targets for killing

The presence of a functional virulence plasmid was also an absolute requirement to sensitize rat lymphoblasts for cytosis by CTLs, since *Y. pseudotuberculosis* strains cured from their virulence plasmid failed to sensitize those blast targets for killing upon in vitro infection (Figs. 1, A and B, 6, and 8A). The lack of cytosis of targets infected with plasmid-cured strains could indicate that proteins encoded by the corresponding plasmidic genes are necessary for interaction of *Y. pseudotuberculosis* with blast targets. Indeed, the surface of all blast cells infected with wild-type *Y. pseudotuberculosis* YPIII (pYV<sup>+</sup>) and tested for lysis of LEW lymphoblast targets. Targets were infected in vitro with wild-type *Y. pseudotuberculosis* strain IP2777 (pYV<sup>+</sup>; solid symbols) or uninfected (open circles). Infected targets were left untreated (solid circles) or were treated with 5 μg/ml CCD during infection (solid squares) or during the chromium release test (CRT; solid diamonds) or with 100 μg/ml gentamicin, which was added throughout target infection (solid triangles). Similar results were observed in two separate experiments.

### FIGURE 3.

Anti-*Y. pseudotuberculosis* CTL effectors are CD8<sup>+</sup> T cells and lyse infected lymphoblast targets in a class I-restricted fashion. A, CTL effectors were generated from LEW rats infected intragastrically with wild-type *Y. pseudotuberculosis* strain YPIII (pYV<sup>+</sup>) and tested for lysis of LEW lymphoblast targets infected with strain YPIII (pYV<sup>+</sup>; solid bars) or uninfected (hatched bars). Immunomagnetic sorting was performed to negatively select for CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells that were compared with unsorted T cells. This experiment was repeated twice with similar results. B, CTL effectors generated as described above were tested for lysis of infected LEW lymphoblast targets that were preincubated with 2, 5, 15, or 30 μg/ml of anti-class I (F16-4-4-11), anti-class II (OX6), or control (υC11) mAb at an E:T cell ratio of 33. Maximal percent specific lysis in this experiment was 23%. Percent inhibition = [1 − (% lysis with mAb/% lysis without mAb)] × 100. This experiment is representative of two.

### TABLE III. Entry of *Y. pseudotuberculosis* into LEW rat lymphoblast targets

<table>
<thead>
<tr>
<th>Strains</th>
<th>% Intracellular Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPIII(pYV&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>0.0002 ± 0.0002&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>YPIII(c:pYV&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>0.008 ± 0.0003</td>
</tr>
<tr>
<td>IP2777(pYV&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>0.003 ± 0.002</td>
</tr>
<tr>
<td>IP2777(c:pYV&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>0.007 ± 0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup>Rat lymphoblast cells (10<sup>6</sup>) were infected in vitro with live *Yersinia* at an infection ratio of 50 bacteria/cell. After 120 min at 37°C, gentamicin (100 μg/ml) was added to the cells and cultures were incubated 60 min further at 37°C to kill extracellular bacteria. Blast cells were lysed by Triton X-100, and viable counts were determined. Strains are as described in Table I. This experiment was repeated twice with similar results.

<sup>b</sup>Mean ± SD for triplicate assays.
actual antigenic targets for *Yersinia*-specific CTLs, that those proteins are required for efficient class I presentation of *Yersinia* Ags, or both.

The level of cytotoxicity against infected targets was similar whether targets were sensitized with *Y. pseudotuberculosis* cultured overnight at 28°C in LB medium or in conditions inducing Yops secretion in the culture medium (e.g., subculture at 37°C in Ca²⁺-deficient medium; not shown). Hence, we speculated that the secretion apparatus of *Yersinia* would allow effector Yops to be presented in a class I-restricted fashion by delivering such proteins directly into the target cytoplasm. To examine the contributions of several Yops encoded by *Yersinia* virulence plasmid to the CTL response, we tested this response against blast targets sensitized with a series of isogenic *Y. pseudotuberculosis* mutants selectively defective for expression of those proteins (Figs. 6 and 8). Targets sensitized with the YP15 deletion mutant of YopH (Fig. 6) were killed at similar levels as targets infected with wild-type *Y. pseudotuberculosis* strain YPIII (pYV¹). Similar results were observed with any of two ypkA mutants (e.g., YPIII(pIB43) insertion mutant, in which the YpkA C-terminal 548–731 amino acid residues are deleted and the YopJ production is abolished, and YPIII(pIB44) in-frame deletion mutant, in which amino acids 207–388 of YpkA are deleted; Fig. 8A). Large differences were observed with the two yopE mutants tested. Targets sensitized with the YPIII(pIB518) mutant of *Y. pseudotuberculosis* producing a nonfunctional truncated form of YopE (deletion of the C-terminal 91 amino acid residues) were killed at levels comparable to those of targets infected with wild-type strain (Fig. 8, A and B), whereas targets sensitized with the YPIII(pIB522) complete deletion mutant of YopE were not killed (Fig. 8B).

Finally, sensitizing targets with isogenic mutants of YPIII deficient in YopB, YopD, or YopB and YopD (both proteins are critically involved in Yops translocation through eukaryotic plasma membrane) completely abolished the CTL response (Fig. 8A).

**Discussion**

This study demonstrates for the first time that infection of rats with one of the three *Yersinia* species pathogenic for humans, *Y.
Y. pseudotuberculosis, elicits a CD8$^+$ T cell-mediated cytotoxic response. Furthermore, this response appears to depend on the RT1-A locus that is a major locus for conventional class I presentation in rats (42). Previous work suggested the possibility that such a class I-restricted T cell response would occur in the setting of infection with *Yersinia* (15). However, a demonstration that such a prediction actually occurred in vivo had not yet been provided.

One of the major issues regarding the possibility to mount a class I-restricted response against extracellular bacteria such as *Y. pseudotuberculosis* is the capacity of eukaryotic cells to process those bacteria components for class I presentation. The extracellular survival of *Yersinia* in the host environment is thought to result from a virulence plasmid-encoded apparatus of Yop secretion, by which *Y. pseudotuberculosis*, can neutralize phagocytic cells (8). Once *Yersinia* attach to the target cell, the secretion system is triggered, and Yops are secreted and directly injected into the eukaryotic target cytoplasm. Several of these Yops exert an action inside their target, contributing to the paralysis of phagocytosis by these cells. This system seems to compromise possible processing of *Yersinia* via phagocytosis by professional APCs, a mechanism important for class I presentation in the case of intracellularly living bacteria (43). However, it can lead to an alternate mechanism of class I presentation that would potentially trigger a CTL response. It is indeed likely that Yops that have access to the cytoplasm can be presented through a class I-mediated pathway (15).

In our experiments sensitization of blast target cells required contact with *Yersinia* that failed to invade those cells, suggesting that the whole bacteria were not penetrating the cell. The possibility that soluble proteins released from *Yersinia* were internalized by target cells before being processed and presented by class I molecules is unlikely, since *Yersinia* presentation required the living state of the bacteria, at least upon contact with the target cell, and was not blocked by addition of CCD. Furthermore, sensitizing targets with *Yersinia* cultured in conditions inducing Yops secretion in the culture medium did not enhance cytolysis. Our observations are consistent with involvement of the Yop-secretion apparatus. This secretion system is triggered by tight contact of *Yersinia* with the eukaryotic cell membrane. Indeed, *inv* mutants

**Figure 8.** Sensitization of blast targets for cytolyis by anti-*Y. pseudotuberculosis* CTLs requires the presence of an intact *Yersinia* translocation apparatus and YopE. A, CTL effectors were generated from LEW rats infected with wild-type *Y. pseudotuberculosis* strain YPIII (pYV$^+$) and were tested for lysis of LEW lymphoblast targets infected with YPIII (pYV$^+$), YPIIIc (pYV$^+$), or YPIII isogenic mutants of Yops. B, CTL effectors generated as described above were tested for lysis of LEW lymphoblast targets infected with YPIII (pYV$^+$), YPIII(pIB518) yopE$^{128-219}$ mutant, or YPIII(pIB522) complete deletion mutant of YopE (same experiment as in Fig. 2B). Similar results were observed in two to four separate experiments for each mutant tested.
that failed to bind to blast targets were unable to sensitize those targets either. Although other Yersinia proteins, such as YadA, can substitute for invasin in adhesion to epithelial cells and allow the Yop secretion apparatus to operate (44), this alternate pathway may not be relevant to lymphoblast cells. Indeed, the presence of YadA was not required for blast target sensitization.

The absolute requirement for virulence plasmid to sensitize target cells is also consistent with the involvement of a Yop secretion apparatus. Our observation that mutants deficient in YopB or YopD failed to sensitize blast cells supports this hypothesis, since both proteins are necessary to translocate effector Yops inside eukaryotic cells. Hence, the most likely antigenic targets in our system are effector Yops. Among the effector Yops tested in our experiments, neither YopH, which was previously shown to be associated with a loss of YopE function (44), Therefore, it is unlikely that the effect of YopE that was observed on the CTL response is associated with a loss of YopE function (44). Therefore, it is unlikely that the effect of YopE that was observed on the CTL response is explained by its known interference with eukaryotic cell metabolism (44). Rather, our data indicate the 23-kDa YopE as a potential antigenic target for anti-Y. pseudotuberculosis CTLs in LEW rats.

This study is interested in several aspects. To our knowledge, this is the first direct demonstration that CTLs can be efficiently primed against extracellularly living Yersinia. The mechanism by which the secretion apparatus of Yersinia is involved in this system may indicate that other bacteria displaying type III secretion apparatus, such as Salmonella, enteropathogenic E. coli, Shigella, or Pseudomonas aeruginosa, could also trigger a CTL response (45–48). This newly described system may also suggest original vaccination strategies to prime CD8+ T cells against infectious agents or even tumoral cells, using engineered bacterial vectors. However, we cannot rule out from the experiments presented in this study that Yersinia proteins other than Yops are candidate Ag for class I presentation. This could particularly be the case if professional APCs were used as targets (which could handle Ag differentially than nonphagocytic lymphoblast cells). However, we believe that the system described herein is predominant, since Yersinia infection usually blocks phagocytosis. Finally, one can speculate upon the efficiency of this CTL mechanism in the host’s fight against Yersinia infection. The production of cytokines by activated CD8+ T cells could contribute to increase bactericidal capacities of macrophages. However, it is not obvious that CTL activity displayed against cells that are not actually invaded by Yersinia is an efficacious defense system. On the contrary, this system could be detrimental to the host, by destroying those cells to which only bacteria bind.

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References