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The B Subunit of Shiga Toxin Fused to a Tumor Antigen Elicits CTL and Targets Dendritic Cells to Allow MHC Class I-Restricted Presentation of Peptides Derived from Exogenous Antigens

Nacilla Haicheur,* Emmanuelle Bismuth,† Sophie Bosset,∗ Olivier Adotevi,∗ Guy Warnier,‡ Valérie Lacabanne,§ Armelle Regnault,§ Catherine Desaymard,† Sebastian Amigorena,§ Paola Ricciardi-Castagnoli,¶ Bruno Goud,† Wolf H. Fridman,∗ Ludger Johannes,‡ and Eric Tartour2,3,*

Immunization with peptide or recombinant proteins generally fails to elicit CTL, which are thought to play a key role in the control of virus-infected cells and tumor growth. In this study we show that the nontoxic B subunit of Shiga toxin fused to a tumor peptide derived from the mouse mastocytoma P815 can induce specific CTL in mice without the use of adjuvant. The Shiga B subunit acts as a vector rather than as an adjuvant, because coinjection of the tumor peptide and the B subunit as separate entities does not lead to CTL induction. We also demonstrated that in vitro the B subunit mediates the delivery of various exogenous CD8 T cell epitopes into the conventional MHC class I-restricted pathway, as this process is inhibited by brefeldin A and lactacystin and requires a functional TAP system. In contrast to other nonviral methods for transport of exogenous Ags into the endogenous MHC class I pathway that involve macropinocytosis or phagocytosis, the Shiga B subunit targets this pathway in a receptor-dependent manner, namely via binding to the glycolipid Gb3. Because this receptor is highly expressed on various dendritic cells, it should allow preferential targeting of the Shiga B subunit to these professional APCs. Therefore, the Shiga B subunit appears to represent an attractive vector for vaccine development due to its ability to target dendritic cells and to induce specific CTL without the need for adjuvant. The Journal of Immunology, 2000, 165: 3301–3308.

The generation of specific CD8+ CTL, which recognize nonapeptides associated with MHC class I molecules, is thought to play a key role in the control of virus-infected cells and tumors. Immunization with peptide alone has occasionally resulted in effective priming of class I-restricted CTL (1–3), but in most cases this procedure failed to induce CTL activity, and in some cases induction of T cell tolerance was observed (4–6). Modification of the natural sequence of peptides to increase their affinity to MHC class I molecules could elicit CTL, but the risk of introducing a bias in the CTL repertoire directed against the natural peptide could not be excluded (7–9). The use of recombinant proteins as immunogens represents an alternative strategy. However, exogenous soluble Ags enter the endosomal pathway and are presented at the cell surface in association with MHC class II molecules, where they activate CD4+ T cells. As a general rule, only peptides derived from cytosolic degradation of cellular proteins bind to MHC class I molecules in the endoplasmic reticulum and are then transported to the cell surface, where they may be recognized by CTL. To overcome this problem, various methods have been developed to target exogenous Ag into the endogenous MHC class I-restricted pathway (10–18). To date, the infectious delivery of Ag into the cytosol using recombinant viral vectors has been successfully demonstrated by several groups and appears to be the most reproducible method to efficiently elicit CTL (19, 20). However, the use of attenuated live virus for vaccination is associated with the risk of causing virus-related disease, especially in immunocompromised patients such as cancer patients and HIV-infected individuals (21, 22). In addition, in recent clinical trials in which patients were immunized with recombinant adenovirus encoding mART-1 or gp100 melanoma Ags, no cellular response against these tumor Ags was recorded (23). This may be due to pre-existing neutralizing anti-virus Abs or interdeterminant competition with peptides derived from the vector (24). Therefore, synthetic vectors must be developed for future human cancer vaccines. In a previous study we showed that a CD8 human tumor Ag fused to the B subunit of Shiga toxin, a nontoxic homopentameric protein responsible for toxin binding to and internalization into target cells by interacting with the glycolipid Gb3 (25), could efficiently be presented in an HLA class I-restricted manner to specific CTL (26). This result was independently confirmed by another study that demonstrated that Shiga holotoxin, carrying a defined peptide epitope from influenza virus, could deliver the Ag into the MHC class I intracellular pathway (27). In the present work we show that...
the Shiga toxin B subunit fused to a tumor peptide derived from the mouse mastocytoma P815 induces the generation of peptide-specific CTL in mice. In addition, we found that the B subunit targets dendritic cells to allow receptor-dependent transport of exogenous peptide into the conventional MHC class I pathway.

Materials and Methods

Mice

Female C57BL/6 (H-2b) and DBA/2 (H-2b) mice from Iffa Credo (L’Arbresle, France) were used between 6 and 8 wk of age. Female TAP−/− bred onto a C57BL/6 background were obtained from Center National de la Recherche Scientifique (Orleans, France).

Recombinant Shiga B fusion proteins and peptide

The Shiga B-P815A fusion protein was constructed by inserting a PCR cassette containing the P815A sequence into the NotI site of pB-Glyc-KDEL (28). The PCR primers were 5’-ATGAAAAAGGGCG CGCCGGTCCATTCTATGGGTGGTCTG-3’ and 5’-ATG AGTCTAGGCGGGCGGGGAAGACGACGCCCCACTTAGATAAGG-3’. The B-Shiga fusion protein was constructed by inserting a PCR cassette containing the SL8 sequence derived residues 257–264 of the full-length OVA into the EcoRI and NotI sites of the B-Eco-Not-modified vector. The PCR primers for the SL8 cassette were 5’-ATGAAAAAGGGCG CGCCGGTCCATTCTATGGGTGGTCTG-3’ and 5’-ATG AGTCTAGGCGGGCGGGGAAGACGACGCCCCACTTAGATAAGG-3’.

Bone marrow-derived dendritic cells (BM-DC) were prepared as previously described (34). Marked induction of P815A-specific CTL was elicited (Fig. 1A). For the P815A-L1-restricted presentation, the cells were plated in 24-well flat-bottom microplates at 5 × 10⁴ cells/well, pulsed at 37°C with Ag, and cocultured for 24 h with 3 × 10⁴ anti-P815 A CTL obtained from mice immunized with the highly immunogenic L1210-P1A-B7 leukemia cell line. The supernatants were harvested, and IFN-γ was measured by ELISA (PharMingen, San Diego, CA).

Flow cytometric analysis

Cells were incubated for 30 min at 4°C with recombinant Shiga B coupled to 5-(4,6-dichlorotriazin-2-yl)fluorescein (DTAF; Sigma). After washes, the analyses were performed on a FACSScan 440 (Becton Dickinson, Mountain View, CA).

Cytotoxicity assay

Cytotoxicity was performed as previously described (26).

Immunization protocols

Female DBA/2 (H-2b) mice (aged 6–8 wk) from Iffa Credo were immunized by i.p. injection on days 0, 8, and 15 with 40 μg of purified recombinant Shiga B protein fused with the P815A peptide and mixed, or not, with IFA. Eight days later spleen cells were stimulated in vitro with L1210-P1A-B7, and CTL activity was measured 5 days later on target P815 or P1-204 with a P815A-negative variant of the P815 mastocytoma.

Results

P815A-specific CTL activity in mice immunized with Shiga B-P815A

We chose to fuse the immunodominant epitope of P1A protein, P815A, to the Shiga B subunit because this Ag represents a murine model for the human tumor Ags belonging to the Mage family. They share the properties of being expressed in various tumors and of being silent in normal adult tissue, except in testis and placenta (35, 36).

DBA2 mice were immunized three times by i.p. injection with Shiga B-P815A in the presence or the absence of IFA. After a secondary in vitro stimulation of spleen cells with the syngeneic leukemia L1210 cells cotransfected with the cDNA encoding P1A and the B7-1 molecules, we demonstrated a significant cytotoxicity of spleen cells against the P815 mastocytoma cells expressing the P1A protein, even in the absence of adjuvant during priming. In contrast, no CTL activity was elicited when mice were immunized with peptide alone or mixed with Freund’s adjuvant (Fig. 1 and data not shown).

To confirm the specificity of these results, we used a P815A-negative variant of P815, P1-204, as a control and subtracted the cytotoxicity measured against P815 and P1-204, as previously described (35). Marked induction of P815A-specific CTL (>20% cytotoxicity) was again demonstrated in mice immunized with the highly immunogenic L1210-P1A-B7 cells or with Shiga B-P815A in the presence or the absence of IFA (Fig. 1B).

Interestingly, the Shiga B subunit did not act as an adjuvant like other toxins (37), but, rather, acted as a vector to target exogenous Ag in the MHC class I pathway. Indeed, when we mixed the P815A peptide with the Shiga B subunit alone, no CTL induction was elicited (Fig. 1C).

Shiga B targets exogenous Ags into the MHC class I pathway

BM-DC from DBA/2 mice (H-2b) were incubated with the Shiga B-P815A fusion protein. As shown in Fig. 2A, the BM-DC then efficiently presented the P815A peptide in a dose-dependent manner to specific anti-P815A CTL obtained after immunization of mice with the L1210-P1A B7 leukemic cell line. As a control, we showed that CTL or BM-DC pulsed separately with the fusion protein alone did not produce IFN-γ (data not shown). The specificity of the anti-P815A CTL was controlled by their ability to
lyse BM-DC only when the target cells were pulsed with the P815A peptide (Fig. 2A).

Using another fusion protein composed of the Shiga B subunit and the immunodominant K\(^b\)-restricted SL8 peptide derived from OVA, we further showed that the D1 dendritic cells (H-2\(^b\)) sensitized in vitro with this construction were able to present the SL8 peptide to B3Z, an anti-SL8 specific T cell hybridoma (Fig. 2B). After washings the cells were cocultured with the specific anti-SL8 B3Z hybridoma (2 \(\times\) 10\(^5\)) carrying a lacZ construct driven by NF-AT elements of the IL-2 promoter. A colorimetric assay with O-nitrophenyl \(\beta\)-D-galactopyranoside as substrate was used to detect \(\beta\)-galactosidase activity in B3Z lysates, reflecting T cell hybridoma activation. In some experiments, the APCs were fixed in 3% paraformaldehyde before sensitization with Ag.

**FIGURE 1.** Induction of CTL response in mice after immunization with Shiga B subunit fused to the P815A peptide. A, DBA2 mice were immunized by i.p. injection on days 0, 8, and 15 with 40 \(\mu\)g (1 nmol) of purified recombinant Shiga B subunit fused with the P815A peptide either alone or mixed with IFA. On day 24, spleen cells were stimulated in vitro with the leukemic cell line L1210 cotransfected with cDNAs encoding P1A and B7 (L1210-P1A-B7). CTL activity was measured 5 days later on the target mastocytoma P815 expressing the P815A peptide. As positive and negative controls, mice were immunized with the L1210-P1A-B7 leukemic cell line or the P815A peptide (1 nmol). B, Data are expressed as P815A-specific cytotoxicity obtained after subtraction of lysis measured against P815 and P1-204, a P815A-negative variant of the P815 mastocytoma. The cytotoxicity observed on the P815A-negative target was usually low and always <25%. Three mice were included in each immunization group, and the results shown are from one representative experiment of three performed. C, DBA2 mice were immunized by i.p. injection on days 0, 8, and 15 with 40 \(\mu\)g (1 nmol) of purified recombinant Shiga B subunit fused with the P815A peptide or with the Shiga B subunit mixed with the P815A peptide (1 nmol). On day 24 spleen cells were stimulated in vitro with the leukemic cell line L1210 cotransfected with cDNAs encoding P1A and B7 (L1210-P1A-B7). CTL activity was measured 5 days later on the target mastocytoma P815 expressing the P815A peptide. As positive and negative controls, mice were immunized with the L1210-P1A-B7 leukemic cell line or the P815A peptide.

**FIGURE 2.** Dendritic cells present CD8 epitopes derived from Shiga B fusion proteins. A, BM-DC (5 \(\times\) 10\(^3\)) from DBA/2 mice were pulsed at 37°C with various concentrations of Shiga B-P815A protein or peptide P815A and cocultured for 24 h with 3 \(\times\) 10\(^4\) anti-P815A CTL obtained from mice immunized with L1210-P1A-B7. The supernatants were then harvested, and IFN-\(\gamma\) was measured by ELISA. B, D1 dendritic cells (10\(^5\)) were pulsed for 5 h with whole OVA, the K\(^b\)-restricted OVA-derived peptide SL8, or the Shiga B subunit fused to the SL8 peptide. After washings the cells were cocultured with the specific anti-SL8 B3Z hybridoma (2 \(\times\) 10\(^5\)) carrying a lacZ construct driven by NF-AT elements of the IL-2 promoter. A colorimetric assay with O-nitrophenyl \(\beta\)-D-galactopyranoside as substrate was used to detect \(\beta\)-galactosidase activity in B3Z lysates, reflecting T cell hybridoma activation. In some experiments, the APCs were fixed in 3% paraformaldehyde before sensitization with Ag.
B-SL8 (Fig. 2B), whereas exogenous synthetic SL8 peptide incubated with the fixed D1 cell line still activated the specific B3Z hybridoma (Fig. 2B). These results confirm our previous experiments on the role of internalization in the MHC class I-restricted presentation of exogenous Ag via the B subunit (26).

Because the in vitro expansion of anti-SL8 T cell hybridoma is much easier than that of anti-P815A CTL, we selected the OVA model for the next experiments.

**Delivery of exogenous Ag into the MHC class I pathway via the B subunit of Shiga toxin is receptor dependent**

In most examples in which synthetic vectors have been used to deliver exogenous peptide in the cytosol, phagocytosis or macropinocytosis was involved in this process, and this MHC class I presentation pathway appeared to require a high Ag concentration (38, 39). In another model the adenylate cyclase toxin of Bordetella pertussis was used to introduce exogenous Ag into the cytosolic pathway. No specific cellular receptor was associated with this process (40). In the case of Shiga toxin it has been clearly established that the B subunit binds specifically to the glycosphingolipid globotriaosylceramide, Gb3 (CD77) (25). Therefore, we tested whether this receptor was involved in B subunit-dependent Ag presentation.

In the first experiment we showed that an excess of the Shiga B subunit not coupled to Ag inhibited presentation of the SL8 peptide delivered via the Shiga B-SL8 fusion protein (data not shown), which suggests a saturable internalization mechanism.

We then demonstrated that the use of an anti-Shiga B IgG or Fab derived from the same Ab, which inhibits binding of the B subunit to Gb3, significantly decreased presentation of the SL8 peptide vectorized by the Shiga B subunit. An isotype control IgG did not interfere with presentation of the SL8 peptide derived from the Shiga B-SL8 fusion protein (Fig. 3A).

In another experiment we cultured dendritic D1 cells for 6 days with PPMP, an inhibitor of Gb3 synthesis (41). Marked down-regulation of Gb3 expression at the plasma membrane on the D1 cell line was observed (Fig. 3B), whereas no modification of membrane expression of the K* class I molecule was detected (data not shown). The D1 cells treated with PPMP and sensitized with the Shiga B-SL8 protein lost the capacity to present the SL8 peptide, whereas exogenous synthetic SL8 peptides incubated with PPMP-treated cells activated the specific T cell hybridoma (Fig. 3C).

Taken together, the above experiments strongly suggest that the Gb3 receptor is involved in the Shiga B subunit-dependent targeting of exogenous peptides into the MHC class I pathway. In addition, in humans (26) as well as in the present study in mice a clear correlation was found between the expression of Gb3 on a given cell and its ability to present a peptide derived from Shiga B fusion protein. Indeed, in the mouse, the dendritic cell line D1 and BM-DC express the Gb3 receptor and present peptides from Shiga (25).

**Analysis of the intracellular pathway leading to MHC class I-restricted presentation of exogenous Ag via the Shiga B subunit**

**TAP requirement for SL8 presentation after pulsing D1 cells with Shiga B-SL8.** Fig. 5A shows that BM-DC from wild-type C57BL/6 mice were efficient in class I-restricted presentation of SL8 processed from Shiga B-SL8, whereas BM-DC from TAP-deficient mice did not present the SL8 epitope after sensitization with Shiga B-SL8 (Fig. 5A). In wild-type and TAP-deficient C57BL/6 mice, direct presentation of synthetic SL8 peptide was equivalent (Fig. 5B). In addition, wild-type and TAP-deficient mice expressed similar levels of Gb3 receptor (data not shown).
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Shiga B-SL8 processing is BFA and lactacytin sensitive. Brefeldin A, which has been shown to inhibit the export of newly assembled MHC class I-peptide complexes from the endoplasmic reticulum to the plasma membrane, completely inhibited presentation of the SL8 peptide derived from Shiga B-SL8 (Fig. 5B). The presence of lactacytin, a specific proteasome inhibitor, during incubation of the D1 cells with soluble Shiga B-SL8 fusion protein also prevented presentation of the CD8 T cell epitope, SL8 (Fig. 5C).

As a control of these experiments, we showed that direct presentation of the synthetic exogenous peptide SL8 was not affected by any of these drugs, which suggests that the Ag-presenting ability of these cells was maintained (Fig. 5, B and C). Finally, chloroquine, which raises the pH of endosomes and therefore inhibits endosomal proteolysis, did not interfere with the presentation of SL8 derived from Shiga B-SL8 (data not shown).

Discussion

We have shown that immunization of mice with recombinant non-toxic Shiga toxin subunit fused to the tumor Ag P815A reproducibly elicits CTL against this Ag. The induction of CTL was observed even in the absence of adjuvant, which distinguishes this vector from other toxins or related agents (42, 43). This may be of particular interest for future human clinical applications in which few adjuvants have been authorized. As reported by other groups, we have shown that treatment of dendritic cells with TNF-α or IL-12 could break the natural tolerance to this Ag (45–47).

Using this model Ag, treatment of mice with recombinant adenovirus or immunization with naked DNA was shown to prime the cytolytic T cell response in mice (48, 49). However, the safety and efficacy of these immunization procedures in humans remain to be established.

To better evaluate the potential role of the Shiga B subunit as a vaccination tool, we demonstrated that in vitro the B subunit was able to mediate the delivery of various exogenous CD8 T cell epitopes into the MHC class I-restricted pathway. These observations confirm and extend previous results (26, 27). In contrast to other methods used to transport exogenous Ags into the endogenous class I pathway, which involve fluid phase endocytosis (40) or phagocytosis (38, 50), we have shown that this process is receptor dependent when the Shiga B subunit is used as a vector of exogenous Ags, because 1) an anti-Shiga B mAb, which inhibits binding of the B subunit to the Gb3 receptor, significantly decreased presentation of exogenous peptide vectorized by the Shiga B subunit (Fig. 3A); 2) when expression of the Gb3 receptor was inhibited on APCs, the ability of these cells to present exogenous peptide delivered by the Shiga B subunit was lost (Fig. 3B); and 3) we have previously noted, as confirmed in the present study, a clear correlation between expression of the Gb3 receptor on a given cell and the possibility to target, via the Shiga B subunit, exogenous Ag into the MHC class I pathway (26).

This receptor-dependent mechanism offers several advantages in terms of sensitivity and targeting specificity compared with previous approaches, as, when using particulate Ags, the introduction of foreign Ags via phagocytosis into the MHC class I pathway proved to be of low efficiency (38), whereas very low Ag concentrations are required to allow MHC class I presentation of exogenous Ag via the Shiga B subunit. However, some groups also demonstrated that the use of live recombinant bacteria could efficiently allow the delivery of exogenous Ag to the MHC class I pathway via phagocytosis (51).

In other models in which toxins have been used to translocate exogenous proteins into the cytoplasm, no cell type specificity could be demonstrated, and many APCs may be the targets of the toxins (40, 52, 53). The Shiga toxin receptor, globotriaosylceramide (Gb3), is a neutral glycosphingolipid that was first identified as an Ag of Burkitt’s lymphoma and other tumors (54, 55). Some human epithelial and endothelial cells and a subset of B lymphocytes located in germinal centers express Gb3 (55–57), while most other cell types are Gb3 negative. We have also demonstrated in mice that spleen and bone marrow cells do not express significant levels of Gb3. Therefore, high Gb3 expression on BM-DC and various dendritic cells should preferentially target the Shiga B subunit to these professional APCs, which play a key role in the initiation of T cell-mediated immune responses (58). The analysis of the presence of Gb3 on subsets of dendritic cells is underway, but we have shown that treatment of dendritic cells with TNF-α increases the expression of Gb3 (data not shown).

Two mechanisms have been proposed to explain how exogenous Ags can be processed and presented in a MHC class I-restricted manner. In some cases the exogenous Ags seem to join the conventional MHC class I pathway, including cytosolic processing by proteasomes and transport of processed peptides into the lumen of the endoplasmic reticulum by the ATP-dependent transporter (TAP). In the endoplasmic reticulum these peptides associate with nascent MHC class I molecules and β2-microglobulin (34, 40, 53, 59). In other cases a TAP-independent pathway has been described in which Ag is processed in endosomes, and the resulting peptides bind to a small fraction of class I molecules that have entered the MHC class II compartments (60–62). Inhibition studies with brefeldin A and lactacytin and the experiments with BM-DC obtained from TAP knockout mice demonstrate that the Shiga B subunit delivers exogenous peptides into the conventional MHC class I pathway. This mode of processing seems more suitable for vaccination purposes, because protein degradation by endosomal proteases might generate peptide fragments that differ from natural...
cytosolic degradation products derived from tumor or viral Ags. This is exemplified by a recent elegant experiment in which the generation of immunodominant class I peptides obtained with Hbs Ag lipoprotein particles processed in the endocytic pathway was compared with the cytosolic generation of peptides after transfection of plasmid DNA encoding Hbs Ag. Distinct MHC class I binding peptides derived from the same Hbs Ag were produced (63).

FIGURE 5. Analysis of the intracellular pathway leading to the MHC class I-restricted presentation of exogenous Ag via the Shiga B subunit. On day 7 of in vitro cell culture BM-DC obtained from either wild-type (WT) or TAP knockout mice were pulsed for 5 h with various concentrations of Shiga B-SL8 (A) or synthetic SL8 peptide (B). After washings the cells were cocultured with the specific anti-SL8 B3Z hybridoma. Activation of the hybridoma was revealed as described in Fig. 2. D1 dendritic cells were preincubated for 1 h with brefeldin A (C) or lactacystin (D) at various concentrations before being pulsed in the presence of the drugs with the Shiga B-SL8 fusion protein or synthetic SL8 peptides. After washings the cells were cocultured with the specific anti-SL8 B3Z hybridoma without drug addition. Activation of the hybridoma was revealed as described in Fig. 2.
In conclusion, the Shiga B subunit appears to represent an attractive candidate for vaccine development due to its ability to target dendritic cells and to induce specific CTL without the need for adjuvant.

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