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Suppression of an Already Established Tumor Growing through Activated Mucosal CTLs Induced by Oral Administration of Tumor Antigen with Cholera Toxin

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Priming of CTLs at mucosal sites, where various tumors are originated, seems critical for controlling tumors. In the present study, the effect of the oral administration of OVA plus adjuvant cholera toxin (CT) on the induction of Ag-specific mucosal CTLs as well as their effect on tumor regression was investigated. Although OVA-specific TCRs expressing lymphocytes requiring in vitro restimulation to gain specific cytotoxicity could be detected by OVA peptide-bearing tetramers in both freshly isolated intraepithelial lymphocytes and spleen cells when OVA was orally administered CT, those showing direct cytotoxic activity without requiring in vitro restimulation were dominantly observed in intraepithelial lymphocytes. The magnitude of such direct cytotoxicity at mucosal sites was drastically enhanced after the second oral administration of OVA with intact whole CT but not with its subcomponent, an A subunit (CTA) or a B subunit (CTB). When OVA plus CT were orally administered to C57BL/6 mice bearing OVA-expressing syngeneic tumor cells, E.G7-OVA, in either gastric tissue or the dermis, tumor growth was significantly suppressed after the second oral treatment; however, s.c. or i.p. injection of OVA plus CT did not show any remarkable suppression. Those mucosal OVA-specific CTLs having direct cytotoxicity expressed CD8αβ but not CD8αα, suggesting that they originated from thymus-educated cells. Moreover, the infiltration of such OVA-specific CD8α T cells was observed in suppressed tumor tissues. These results indicate that the growth of ongoing tumor cells can be suppressed by activated CD8αβ CTLs with tumor-specific cytotoxicity via an orally administered tumor Ag with a suitable mucosal adjuvant. The Journal of Immunology, 2008, 180: 4000–4010.

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3 Abbreviations used in this paper: IEL, intraepithelial lymphocyte; CT, cholera toxin; CTA, A subunit (toxic); CTB, B subunit (nontoxic); DC, dendritic cell; GM, monosialoganglioside; Hps, Helicobacter pylori; LPL, lamina propria lymphocyte; MadCAM-1, mucosal addressin cell-adhesion molecule-1; OVA-CT, CT-conjugated cholera toxin; OVA-CTA, CT-conjugated CTA subunit (toxic); OVA-CTB, CT-conjugated CTB subunit (nontoxic); P18, OVA peptide (P18); rVV, recombinant vaccinia virus; rVV-P18, recombinant vaccinia virus-P18; Tg, transgenic; TIL, tumor-infiltrating lymphocyte; TCR, T-cell receptor.

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CD4+ T cell responses in the spleen, reflecting the systemic compartment, and in Peyer’s patches (PPs), reflecting the mucosal compartment (17). In addition, it has been demonstrated (18) that OVA-specific CTLs could be primed in C57BL/6 mice following oral exposure to a combination of OVA with CT, and specific cytotoxic activity was detected from spleen cells (SCs) only when they were restimulated in vitro with irradiated OVA-expressing syngeneic tumor cells, E.G7-OVA, which are OVA gene-transfected EL4 thymoma cells (19, 20). Also, intranasal preimmunization with OVA peptide (SIINFEKL) plus CT primed similar OVA-specific CTLs in the spleen of C57BL/6 mice, and the immunized mice were protected from the development of transferred E.G7-OVA (21).

Moreover, it has been shown that adoptive transfer of naïve CD8+ OVA-specific OT-I T cells into E.G7-OVA tumor-bearing syngeneic mice did not inhibit tumor growth, although adoptive transfer of preactivated OT-I CTL in vitro inhibited tumor growth (22). Furthermore, it has recently been reported that vaccination with dendritic cells (DCs) prepsulsed ex vivo with CT-conjugated OVA (OVA-CT) gave rise to OVA-specific splenic CD8+ T cells that produced IFN-γ, were cytotoxic to E.G7-OVA cells in vivo, and rejected already established in vivo E.G7-OVA tumors associated with high numbers of tumor-infiltrating CD8+ T cells (23), indicating that the elimination of previously established tumors might require the infiltration of tumor-specific activated CD8+ CTLs.

In the present study, we found two distinct types of CD8αβ-positive T cells among freshly isolated lymphocytes expressing OVA-specific TCRs, which can be detected by OVA peptide-bearing tetramers. One is in an activated effector state with cytotoxic activity and the other is a resting state and may gain cytotoxicity when stimulated with an OVA epitope peptide in vitro. Based on the observations, we defined direct cytotoxicity as the former state, and CD8αβ T cells that produced IFN-γ, were cytotoxic to E.G7-OVA cells in vivo, and rejected already established in vivo E.G7-OVA tumors associated with high numbers of tumor-infiltrating CD8+ T cells (23), indicating that the elimination of previously established tumors might require the infiltration of tumor-specific activated CD8+ CTLs.

The spleen was aseptically removed and a single cell suspension was prepared. For osmotic hemolysis, single cells were suspended in 0.1 M sucrose and PBS was added immediately. To enrich IELs, the obtained guts were inverted and cut into several segments that were transferred to a 50-ml conical tube (Becton Dickinson Labware) containing 45 ml of HBSS with 5% FCS, 100 U/ml penicillin (Invitrogen Life Technologies), and 100 μg/ml streptomycin (Invitrogen Life Technologies). The tube was then shaken at 37°C for 45 min (horizontal position; orbital shaker at 150 rpm). Harvested cells from the intestinal epithelium were transferred through a 10-ml syringe column containing loosely packed glass wool to remove tissue debris. Subsequently, the cells were suspended in 30% Percoll solution (Amersham Biosciences) and centrifuged for 20 min at 1,800 rpm. Cells at the bottom of the solution were then subjected to Percoll discontinuous gradient centrifugation for 20 min at 1,800 rpm and IELs were recovered at the interface of 44 and 70% Percoll solutions.

Flow cytometry analysis

Cells were double-stained with PE-labeled H-2Kb/OVA tetramer-SIINFEKL (Beckman Coulter) or H-2Kb/PE1 tetramer-SSYRPRGVL (Medical & Biological Laboratories) and FITC-labeled mouse TCRαβ, CD8α, CD8β (BD Pharmingen), or CD11b (CaluBio). Experiments were performed according to guidelines for the care and use of laboratory animals set by the National Institutes of Health (NIH; Bethesda, MD) and approved by the Review Board of Nippon Medical School (Tokyo, Japan).

Oral and systemic immunization

Chicken egg OVA, grade V (Sigma-Aldrich), was dissolved in sterilized PBS. Mice were orally administered 100 μg of OVA or 10 μg of CT (List Biological Laboratories) alone or 100 μg of OVA plus 10 μg of CT, or CTB (List Biological Laboratories) in 0.3 ml of PBS. In some experiments, mice were orally administered 10 mg of OVA plus 10 μg of CT. For systemic immunization, mice were i.p. or s.c. injected with 100 μg of OVA or the same dose of OVA plus 10 μg of CT.

Preparation of IELs, lamina propria lymphocytes (LPLs), SCs, and tumor-infiltrating lymphocytes (TILs)

IELs were prepared by the method described previously (12). In brief, after the small intestine, large intestine, or stomach was dissected from mice, fecal materials were flushed from the lumen with HBSS (Invitrogen Life Technologies) and connective tissues were carefully removed. The obtained guts were transferred into several segments that were transferred to a 50-ml conical tube containing 45 ml of HBSS with 5% FCS and 1 mM EDTA (Wako Pure Chemical Industries). The tube was shaken at 37°C for 45 min (horizontal position; orbital shaker at 150 rpm). Harvested cells from the intestinal epithelium were transferred through a 10-ml syringe column containing loosely packed glass wool to remove tissue debris. Subsequently, the cells were suspended in 30% Percoll solution (Amersham Biosciences) and centrifuged for 20 min at 1,800 rpm. Cells at the bottom of the solution were then subjected to Percoll discontinuous gradient centrifugation for 20 min at 1,800 rpm and IELs were recovered at the interface of 44 and 70% Percoll solutions. LPLs were prepared by the method described previously (24). In brief, after the small intestine, large intestine, or stomach was dissected from mice, fecal material was flushed from the lumen with HBSS and PPs were carefully removed. The obtained guts were transferred into several segments that were transferred to a 50-ml conical tube containing 45 ml of HBSS with 5% FCS and 1 mM EDTA (Wako Pure Chemical Industries). The tube was shaken at 37°C for 45 min (horizontal position; orbital shaker at 150 rpm). The gut segments were then washed with PBS and shaken in 40 ml of HBSS with 5% FCS and 0.1 mg/ml collagenase (Sigma-Alrich) at 37°C for 45 min (horizontal position; orbital shaker at 60 rpm). Harvested cells were passed through a nylon mesh and suspended in 40% Percoll solution, and then 70% Percoll solution was underlain. The solution was centrifuged for 20 min at 1,800 rpm and LPLs were recovered at the interphase of 40 and 70% Percoll solutions. These procedures provided >95% viable lymphocytes with a cell yield of 5–10 x 106 of small intestinal IELs, 2–3 x 106 of large intestinal IELs, 7–12 x 106 of gastric IELs, 4–9 x 106 of small intestinal LPLs, 1–3 x 106 of large intestinal LPLs, or 5–9 x 106 of gastric LPLs per mouse. The cells were suspended in complete T cell medium (25) composed of RPMI 1640 medium (Sigma-Aldrich) supplemented with 2 mM l-glutamine (ICN Biomedicals), 1 mM sodium pyruvate (Invitrogen Life Technologies), 0.1 mM nonessential amino acid (Invitrogen Life Technologies), a mixture of vitamins (ICN Biomedicals), 1 mM HEPES (Invitrogen Life Technologies), 100 U/ml penicillin (Invitrogen Life Technologies), 100 μg/ml streptomycin (Invitrogen Life Technologies), 50 μM 2-ME (Sigma-Alrich), and heat-inactivated 10% FCS. For TIL preparation, tumors were removed from mice, incubated in 1 mg/ml collagenase (Wako Pure Chemical Industries) with PBS at 37°C for 1 h, and crushed gently. TILs were prepared using Percoll solutions as described in the previous paragraph regarding IEL preparation. The spleen was aseptically removed and a single cell suspension was prepared. For osmotic hemolysis, single cells were suspended in 0.1 X PBS and an equal amount of 2 X PBS was added immediately. To enrich IELs, LPLs, and TILs from mice, the interface between the 40 and 70% Percoll solutions (26), in which NK cells and unfractonated SCs, which may also include NK cells, must be included, was collected.

In vitro restimulation of SCs or IELs with E.G7-OVA

Lymphocytes were restimulated in vitro by the method described previously (19). Freshly isolated SCs (3 x 105) or IELs (3 x 105) were restimulated to a 3 x 106 population (20,000 rad) E.G7-OVA cells (19, 20) (22 American Type Culture Collection) in 10 ml of complete T cell medium per upright 25-cm2 flask in 5% CO2 at 37°C for 6 days. Six days later, the viability of the lymphocytes was 35–51% in SCs and 16–26% in IELs.

Materials and Methods

**Mice**

Six- to 8-wk-old female C57BL/6 (H-2b) mice were purchased from Charles River Japan, maintained in microisolator cages under pathogen-free conditions, and fed autoclaved laboratory chow and water. All animal experiments were performed according to guidelines for the care and use of laboratory animals set by the National Institutes of Health (NIH; Bethesda, MD) and approved by the Review Board of Nippon Medical School (Tokyo, Japan).

**Oral and systemic immunization**

Chicken egg OVA, grade V (Sigma-Aldrich), was dissolved in sterilized PBS. Mice were orally administered 100 μg of OVA or 10 μg of CT (List Biological Laboratories) alone or 100 μg of OVA plus 10 μg of CT, or CTB (List Biological Laboratories) in 0.3 ml of PBS. In some experiments, mice were orally administered 10 mg of OVA plus 10 μg of CT. For systemic immunization, mice were i.p. or s.c. injected with 100 μg of OVA or the same dose of OVA plus 10 μg of CT.
in vitro restimulated cells were collected and their OVA-specific cytotoxicity was measured by the following procedure.

**CTL assay**

For the CTL assay, freshly isolated IELs, SCs, or TILs were used. Cytolytic activity was measured using a standard 3H-Cr-release assay as previously described (12). In brief, various numbers of effector cells were incubated with $3 \times 10^{5}$ 3HCr-labeled targets for 6 h at 37°C in 200 μl of RPMI 1640 medium containing 10% FCS in round-bottom 96-well cell culture plates (BD Biosciences). After incubation, the plates were centrifuged for 10 min at 330 × g, and 100 μl of cell-free supernatants were collected to measure radioactivity with a Packard Auto-Gamma 5650 counter (Hewlett-Packard Japan). Maximum release was determined from the supernatant of cells that had been lysed by the addition of 5% Triton X-100, and spontaneous release was determined from target cells incubated without added effector cells. The percentage of specific lysis was calculated as $100 \times (\text{experimental release} - \text{spontaneous release})/\text{maximum release}$. SEs of the means of triplicate cultures were always <5% of the mean. Each experiment was performed at least three times.

**Measurement of in vivo antitumor effects**

E.G7-OVA cells (5 × 10⁶), OVA gene-transfected EL4 thymoma cells (19, 20), were implanted into the gastric or dermal tissue of syngeneic C57BL/6 mice (H-2b). For tumor implantation into the gastric tissue, mice were anesthetized and underwent an abdominal operation and then E.G7-OVA cells in 50 μl of RPMI 1640 were injected into the muscle layer of the stomach using a 29-gauge needle (Terumo). For implantation into the dermal tissue, mice were anesthetized and E.G7-OVA cells in 100 μl of RPMI 1640 were injected intradermally using a 29-gauge needle. On day 3 after implantation into the gastric or dermal tissue, when the tumor mass became visible, tumor-bearing mice were orally or systemically administered OVA plus CT as described above. Seven days after the first administration, some of the mice were similarly boosted with the same administration. Tumor volume (V) was calculated according to the formula $V = ab^2/2$ as reported previously (28). When the longer axis of each tumor was >20 mm, all mice were anesthetized and sacrificed according to the guidelines for the care and use of laboratory animals set by the NIH.

**Histological analysis of tumor tissues**

Freshly excised tumor tissues were embedded in Tissue-Tek OCT compound (Sakura Finetek) at −80°C. Tissue sections were sectioned at 6 μm using a cryostat. Sections were placed on a poly-L-lysine-coated glass slide, air dried, and then fixed in 10% formalin PBS for 5 min and stained with H&E. For immunohistochemical staining, sections were fixed in cold acetone for 5 min and incubated with blocking solution (Block-ace; Dainippon Pharmaceutical) for 30 min at 37°C and then incubated with biotin-conjugated rat anti-CD8α Ab (CalTag Laboratories) or control isotype-matched rat IgG2a Ab (CalTag Laboratories) overnight at 4°C. Endogenous peroxidase was quenched by incubation in 3% H₂O₂ and 0.1% NaN₃ in distilled water for 10 min. The sections were incubated with avidin-biotin peroxidase complexes (Vectastain ABC kit; Vector Laboratories) followed by color reaction with aVectastain diaminobenzidine substrate kit (Vector Laboratories).

**Statistical analysis**

Student’s t test was used to determine the statistical significance of differences between groups in tumor growth. Data were considered significant at $p < 0.05$.

**Results**

**Priming of OVA-specific CD8αβ-positive CTLs with direct cytotoxicity via oral administration with OVA plus CT**

It has been reported that OVA-specific CTLs could be primed in C57BL/6 mice by oral or i.v. immunization with OVA plus CT together with nontoxic CTB, and specific cytotoxic activity was detected from immune SCs only when they were restimulated in vitro with irradiated OVA-expressing syngeneic tumor (E.G7-OVA) cells (18). It has also been shown that activated CTLs but not naive primed CTLs could represent antitumor responses in vivo (22). Similarly, we have recently observed in HIV-1-specific CTL-TCR transgenic mice that activated CTLs but not freshly iso-

![FIGURE 1. Analysis of OVA-specific direct cytotoxicities in IELs and SCs after primary immunization with OVA plus CT. A. Analysis of H-2Kb/EL4*OVAp tetramer-positive cells. C57BL/6 mice were orally administered OVA+CT on day 0. IELs and SCs were collected from mice 5 days after the first oral administration, stained with either PE-labeled H-2Kb/EL4*OVAp tetramer-SIINFEKL or H-2Kb/EL4*OVAp tetramer-SSYRRPVGI together with FITC-labeled anti-mouse TCRβ, and analyzed by flow cytometry. Each value represents the percentage of cells expressing both indicated markers. Data are representative of three independent experiments. B. Kinetics of H-2Kb/EL4*OVAp tetramer-positive cells after primary immunization. C57BL/6 mice were orally administered OVA plus CT once. IELs and SCs were collected from mice at various days after the first oral administration, stained with either PE-labeled H-2Kb/EL4*OVAp tetramer together with FITC-labeled anti-mouse TCRβ, CD8α, or CD8β, and analyzed by flow cytometry. The results are shown as the mean ± SD of four mice. C. Kinetics of OVA-specific direct cytotoxic responses. C57BL/6 mice were orally primed and cells were collected as described in B. OVA-specific CTL responses were measured by 3H-Cr-release assay using E.G7-OVA cells (H-2b), YAC-1 cells, and EL4 cells (H-2b) pulsed with or without 4 μM OVA-peptide, SIINFEKL, as target cells. E:T ratio was 100:1. The results shown as the mean ± SD in triplicate of pooled cells from two mice are representative of three independent experiments.

looped TCR-bearing CD8αβ-positive T cells showed specific cytotoxicity, and the most critical sites for activating TCR-bearing CD8αβ T cells were mucosal compartments when Tg mice were administered a specific Ag for TCR (12).

These findings prompted us to examine whether direct OVA-specific cytotoxic activity could be induced among IELs in mice
Expansion of direct OVA-specific cytotoxicities after oral boosting with OVA plus CT. A. Activated H-2Kb/OVA tetramer-positive cells after oral boosting. C57BL/6 mice were orally administered CT, OVA, or OVA plus CT once weekly for 2 wk. IELs and SCs were collected from mice 3 days after the second oral boost, and cocultured with 3 x 10^5 irradiated E.G7-OVA. Six days later, OVA-specific lysis of stimulated IELs and SCs was measured by 51Cr-release assay. The E:T ratio is 100:1. The results shown as the mean ± SD in triplicate of pooled cells from two mice are representative of three independent experiments. B. OVA-specific CTL responses of isolated IELs were measured by 51Cr-release assay using E.G7-OVA cells, YAC-1 cells, and EL4 cells pulsed with or without OVA peptide as targets. The E:T ratio is 100:1. Data are shown as the mean ± SD in triplicate of pooled cells from two mice. The results are representative of three independent experiments for both A and B.

administered OVA plus CT orally without requiring in vitro re-stimulation. To carry out this experiment, we used a H-2Kb/OVA tetramer to detect cells expressing OVA-specific TCR in freshly isolated IELs as well as in the SCs of primed mice 5 days after immunization. Also, to evaluate the purity of IELs, CD103 (integrin

FIGURE 2. Expansion of direct OVA-specific cytotoxicities after oral boosting with OVA plus CT. A. Activated H-2Kb/OVA tetramer-positive cells after oral boosting. C57BL/6 mice were orally administered CT, OVA, or OVA plus CT once weekly for 2 wk. IELs and SCs were collected from mice 3 days after the second oral boost and stained with PE-labeled H-2Kb/OVA tetramer or H-2Kb/PB1 tetramer together with FITC-labeled anti-mouse TCRβ. Each value represents the percentage of cells expressing both indicated markers. Data are representative of three independent experiments. B. OVA-specific CTL responses of isolated IELs were measured by 51Cr-release assay using E.G7-OVA cells, YAC-1 cells, and EL4 cells pulsed with or without OVA peptide as targets. The E:T ratio is 100:1. The results shown as the mean ± SD in triplicate of pooled cells from two mice are representative of three independent experiments. D. Activation of OVA-specific CTLs by in vitro restimulation (Restim.). C57BL/6 mice were orally administered CT, OVA, or OVA plus CT once weekly for 2 wk. IELs (3 x 10^5) and SCs (3 x 10^5) were collected from mice 9 days after the second oral boost, stained with PE-labeled H-2Kb/OVA tetramer together with FITC-labeled anti-mouse TCR β, CD8α, or CD8β, and analyzed by flow cytometry. The results are shown as the mean ± SD of four mice. C. Kinetics of the secondary expansion of OVA-specific direct CTL responses. C57BL/6 mice were treated orally and the cells were collected as described in B. OVA-specific CTL responses were measured by 51Cr-release assay using E.G7-OVA cells, YAC-1 cells, and EL4 cells pulsed with or without OVA peptide as targets. The E:T ratio is 100:1. The results shown as the mean ± SD in triplicate of pooled cells from two mice are representative of three independent experiments. What is the role of CD103 in this experiment? CD103 is used to evaluate the purity of IELs.
α-IEL chain-positive cells in the collected samples were examined by flow cytometry. CD103 is highly expressed on >90% of IELs (29, 30) but on only 15% of SCs (31). In the present study, anti-mouse CD8

B

SCs (Fig. 1

C

), but it did not correspond to NK cell activity as was maximal at day 7 after oral immunization with both IELs and SCs (Fig. 1

D

). The number of tetramer-positive cells, to which the magnitude of direct OVA-specific cytotoxicity corresponded, was maximal at day 7 after oral immunization with both IELs and SCs (Fig. 1

E

), but it did not correspond to NK cell activity as was maximal at day 7 after oral immunization with both IELs and SCs (Fig. 1

F

). The results clearly demonstrate that direct OVA-specific CTL cytotoxicity is dominantly observed in mucosal IELs after primary oral administration of OVA plus CT.

Augmentation and kinetics of direct OVA-specific cytotoxicity by CD8αβ CTLs among IELs and SCs via oral boosting with OVA plus CT at day 7 after the primary administration

As shown above, because only 4.5–5.0% of IELs were temporarily activated by a one-shot oral administration, we extensively examined the effect of oral boosting with OVA plus CT at various days after primary immunization. The number of H-2Kb/OVA tetramer-positive cells was significantly enhanced among IELs but not among SCs when primed mice were boosted (Fig. 2A). Such an effect was highest when mice were boosted at day 7 after initial...
priming (data not shown). Tetramer-positive cells were again TCRβ-, CD8α-, and CD8β-positive IELs and their number peaked at day 3 after boosting (Fig. 2B). Correspondingly, direct OVA-specific cytotoxicity was greatly enhanced among IELs and the maximal cytotoxicity of IELs was observed at day 3 after boosting (Fig. 2C), although such direct cytotoxicity appeared to be completely lost in SCs (Fig. 2C). Nonetheless, SCs showed good epitope-specific cytotoxicity similar to that of IELs when they were restimulated in vitro with irradiated E.G7-OVA (Fig. 2D), suggesting that the priming effect by the oral administration of OVA plus CT also remained in systemic SCs.

It should be noted that the memory of OVA-specific CTLs persisted among IELs but not SCs. When secondary boosting with OVA plus CT was performed even 6 mo after primary boosting at day 7, the number of H-2Kb/OVA tetramer-positive cells was still detected at 6% in IELs, and they showed remarkable direct cytotoxicity of 84.5% against E.G7-OVA cells and 58.4% against EL4 cells pulsed with OVA peptide 3 days after secondary boosting (data not shown). Again, we could not detect any measurable direct cytotoxicity in the SCs of secondary boosted mice (data not shown).

Both CTA and CTB subunits are required to induce direct OVA-specific cytotoxicity in IELs

CT is comprised of a single A subunit, CTA, and five B subunits, CTB. When OVA was administered orally to mice with either 10 μg of CTA or an equal amount of CTB, H-2Kb/OVA tetramer-positive cells as well as direct OVA-specific cytotoxicity could not be detected in IELs (Fig. 3, A and B) and SCs (data not shown), although a significant number of tetramer-positive cells and strong direct OVA-specific cytotoxicity were observed among IELs of mice administered orally with OVA plus 10 μg of intact CT (Fig. 3, A and B). Even when using 50 μg of CTA or CTB for the administration of OVA, direct cytotoxicity was not observed (data not shown); therefore, both CTA and CTB subunits are required to induce direct Ag-specific cytotoxicity.

Effects of oral administration and boosting with OVA plus CT on OVA-expressing tumor growth established in the stomach

We then examined in vivo antitumor effects of oral administration with tumor Ag plus CT on already established tumors growing in mice. C57BL/6 mice were implanted with 5 × 10⁶ syngeneic E.G7-OVA cells into the muscle layer of the stomach (Fig. 4A). Three days later, tumor-bearing mice (Fig. 4A) were orally administered various combinations of OVA plus adjuvant and boosted with the same materials 7 days after the initial oral administration. To our surprise, tumor growth in the stomach of mice orally administered OVA plus CT twice was visually (Fig. 4B) and significantly (p < 0.05; Fig. 4C) suppressed on day 18 after tumor implantation as compared with other control groups such as OVA plus CTB or CT alone. However, when tumor-bearing mice were orally administered OVA plus CT once and without boosting, no statistically significant suppression was observed on day 18 as compared with untreated control mice, although a slight suppressive effect could be seen (Fig. 4D). Therefore, two oral administrations of tumor-Ag plus CT with an appropriate interval induced significant ongoing tumor suppression.

As previously shown, direct OVA-specific cytotoxicity among small intestinal IELs was greatly enhanced after boosting with OVA plus CT (Fig. 2, A, B, and C). We also examined whether direct OVA-specific CTLs were induced in the IELs and LPLs of the stomach, small intestine, and large intestine from boosted mice in which gastric tumor growth was significantly suppressed. We observed an increase in the number of H-2Kb/OVA tetramer-positive...
FIGURE 6. Infiltration of CD8αβ positive lymphocytes into tumor tissues in mice orally administered OVA plus CT. C57BL/6 mice were implanted with 5 × 10^6 E.G7-OVA cells into the muscle layer of the stomach (A, a-h) or skin (B, a-h). Three days later, tumor-bearing mice were orally administered OVA (A, a-d, and B, a-d) or OVA plus CT (A, e-h and B, e-h). Seven days later, the second oral administration was performed in the same manner. Gastric and dermal tumor tissues were removed from mice 3 days after the second oral boost. Frozen sections of tumor tissues and normal tissues were prepared and stained with H&E (A, a, b, e, f, and i and B, a, b, e, f, and i) or immunohistochemically stained with biotin-conjugated rat anti-CD8α mAb (A, c and g, and B, c and g) or control isotype-matched rat IgG2a Ab (A, d and h, and B, d and h). Image magnification is either ×40 (A, a, e, and i and B, a, e, and i) or ×400 (A, b-d and f-h and B, b-d and f-h). A, b and f and B, b and f are enlarged images (×400) of the squared areas in the images (×40) of A, a and e and B, a and e, respectively.

Effects of oral administration and boosting with OVA plus CT on already established OVA-expressing dermal tumor growth

Next, we investigated the effect of the oral administration of tumor Ag plus CT on tumor growth in the skin, where the digestive tract is not directly associated. Mice were implanted with 5 × 10^6 E.G7-OVA cells intradermally. Three days later, tumor-bearing mice were orally administered various combinations of OVA plus adjuvant and boosted with the same materials 7 days after the initial oral administration. Interestingly, intradermal tumor growth was again strongly suppressed visually 11 days after tumor implantation in the dermis of mice orally administered OVA plus CT as compared with various other groups (Fig. 5A). This visual effect was confirmed by calculating the volume of the tumors established at day 11 and day 13 in each group (p < 0.05 and p < 0.005, respectively; Fig. 5B). We also examined the effect of the administration of tumor Ag plus CT via various routes on intradermal tumor growth. Although a slight suppression was observed by s.c. inoculation of OVA plus CT, tumor growth was not suppressed at all by i.p. administration in comparison with the oral treatment group (Fig. 5C). It should be noted that tumor growth in the dermis was markedly suppressed even by a single oral administration of OVA plus CT on day 0, 3, 7, or 10 after tumor implantation (Fig. 5D). In each group, tumor growth was suppressed (p < 0.05) and the tumor volume was small around 7 days after oral administration. Unexpectedly, there was almost no difference in the suppressive effects on tumor growth between mice treated with a single administration and boosted mice showing much stronger direct cytotoxicity (data not shown). However, when the dosage quantity of OVA was decreased by one-tenth, tumor growth in boosted mice was more significantly (p < 0.005) suppressed than in nonboosted mice (p < 0.01; Fig. 5E). Collectively, the results indicate that the oral administration of tumor Ag plus CT with appropriate mucosal boosting may induce a remarkable suppression of already established tumor growth in the skin via mucosally generated CTLs.

Infiltration of CD8αβ-positive cells in suppressed tumor tissues

We thus examined whether OVA-specific CD8αβ-positive CTLs were actually seen in suppressed tumor tissues such as the stomach and dermis. To determine tumor-infiltrating CD8αβ+ cells, immunohistochemical staining was performed using biotin-conjugated rat anti-CD8α mAb (Fig. 6A, c and g and B, c and g) or control isotype-matched rat IgG2a Ab (Fig. 6A, d and h and B, d and h). Indeed, although mononuclear cells were seen in the gastric tumor tissues of mice treated with OVA alone, CD8αβ-positive cells were not observed at all (Fig. 6A, a–d). In contrast, infiltration of inflammatory mononuclear cells together with CD8αβ-positive cells was observed in suppressed gastric tumor tissues (Fig. 6Ag). As shown in Fig. 6Ai, normal gastric tissue is composed of the epithelium, lamina propria, lamina muscularis mucosae, muscle layer, and serosa from the inside surface in sequence. As compared with normal gastric tissue, a great number of large tumor cells (EG7-OVA) were mainly found between the lamina muscularis mucosae and serosa of tumor-implanted mice (Fig. 6A, a and b) and the infiltration of tumor cells into the lamina propria over the lamina muscularis mucosae was also observed (data not shown). However, in suppressed gastric tumor tissues (Fig. 6Ae) the tumor cell layer under the lamina muscularis mucosae was markedly thinner than that of an unsuppressed tumor (Fig. 6Ae), in which

![Image](https://example.com/image1.png)

![Image](https://example.com/image2.png)
Discussion

In the present study we demonstrated that when OVA plus intact CT was orally administered into mice, direct OVA-specific cytotoxicity was predominantly induced in IELs rather than SCs after the first oral priming, and direct OVA-specific cytotoxicity was remarkably expanded in IELs but not in SCs after oral boosting with the same doses of OVA plus CT. Such OVA-specific CTLs were thymic conventional K⁺ class I MHC molecule-restricted TCRαβ⁺ CD8αβ⁺ T cells (32). Moreover, the growth of the OVA-expressing tumor E.G7-OVA thymoma, established previously either in the stomach or dermis, was significantly suppressed by the oral administration of OVA plus CT. Furthermore, marked infiltration of OVA-specific TCRαβ⁺ CD8αβ⁺ CTLs with direct cytotoxicity in reduced tumor tissues was observed. These results suggest that activated CTLs with specific cytotoxicity generated at mucosal compartments by oral administration with OVA plus intact CT may be responsible for already established tumor regression.

The majority of tumor regression studies associated with activation of the immune system have focused on systemic immunity observed in the spleen, lymph nodes, and circulating blood rather than local mucosal immunity seen in gut IELs. Those studies have demonstrated only preventative results for tumor establishment by preadministration of tumor Ag plus a suitable adjuvant. In addition, to our knowledge only one study has been shown to suppress already established tumor growth by activating and expanding tumor infiltrating CD8⁺ CTLs (23). In that study, i.v. vaccination with DCs pulsed ex vivo with OVA-CT at day 3 and boosted at day 10 after OVA-expressing E.G7 tumor injection induced complete rejection of a visible tumor within 3 wk after the first treatment. Although the inoculation route and the materials for vaccination were different from ours, the timing of the priming and boosting to induce the suppression of already established tumor growth correlated exactly, suggesting that their methods may also initiate strong mucosal direct cytotoxicity mediated through CD8⁺ CTLs.

measurement of tumor-specific cytotoxic activity by tumor-infiltrating cells in tumor-suppressed mice

To confirm whether infiltrated CD8αβ⁺-positive T cells achieved OVA-specific cytotoxicity, we isolated TILs containing both mononuclear cells and CD8αβ⁺-positive T cells from suppressed dermal tumor tissues as well as from their IELs and SCs. As expected, the number of H-2K\(^{b}\)/OVA tetramer-positive cells increased in both the TILs and IELs but not in the SCs of mice bearing suppressed tumors induced by oral administration with OVA plus CT as compared with mice inoculated with OVA plus CT via another route (Fig. 7A), and those increased tetramer-positive cells showed significant direct OVA-specific CTL activity (Fig. 7B). It should be noted that, although the number of increased cells specific for the H-2K\(^{b}\)/OVA tetramer was small in mice inoculated with OVA plus CT s.c., both the TILs (0.8%) and the SCs (0.4%) but not the IELs (0.1%) of the mice represented a detectable level of direct OVA-specific cytotoxicity (Fig. 7B). These findings suggest that s.c. immunization with Ag plus CT may preferably activate systemic (splenic) Ag-specific CTLs rather than local (intraepithelial) CTLs. Moreover, NK cell cytotoxicity determined against YAC-1 cells was not observed in TILs, IELs, and SCs by oral, s.c., or i.p. immunization of OVA plus CT (Fig. 7B), indicating that the suppression of tumor growth was mainly mediated by CD8αβCTLs rather than by NK cell cytotoxicity.
Similar to our findings, they also showed that immunization with OVA-CT but not with CTB-conjugated OVA (OVA-CTB)-prepulsed DCs could successfully induce complete rejection of already established tumor growth, although OVA-CTB-prepulsed DC inoculation prevented tumor establishment but not ongoing tumor growth in the skin. Moreover, they insisted that OVA has to be coupled to CT and should be loaded onto DCs for therapeutic DC vaccination based on the observation that neither OVA-CT nor DCs pulsed with unconjugated OVA plus CT could prevent tumor progression. Nonetheless, our findings shown here apparently indicate that we were able to induce effective suppression of ongoing tumor growth by simple oral administration with unconjugated OVA and CT. These results suggest that we may control already established tumor growth at the surface compartments by activating mucosal CD8+ CTLs via orally administered tumor Ag with a suitable mucosal adjuvant. Also, when OVA-CT is orally administered, the conjugation between OVA and CT may be broken through digestion by enzymes secreted in the gastrointestinal tract. Recently, we have reported that modification of OVA in the gastrointestinal tract is essential for oral tolerance induction against OVA (33). Therefore, it is possible that gastrointestinal digestion or modification of OVA may facilitate the delivery of OVA Ag into DCs, critical APCs for OVA-specific CTL induction.

For the efficient induction of such OVA-specific CTLs in vivo using DCs, Eriksson et al. have reported that OVA-CT-prepulsed DC immunization required at least two DC injections, reflecting the priming/boosting procedure (23); however, we have observed that a single oral administration of OVA plus CT seems sufficient to induce effective CTLs to prevent E.G7-OVA thymoma growth, particularly in the skin. This may be because mucosally crosspresented CTLs through oral immunization may be more potent than systemically crosspresented CTLs to suppress transplanted tumors at the mucosal compartment, and oral administration of OVA plus CT seems more efficient to induce mucosal CTLs than i.v. Ag-loaded DC inoculation. Further studies will be needed to explain the differences.

Although both CT-conjugated-OVA and CTB-conjugated OVA are cross-presented by MHC class I in DCs, only CT-OVA but not CTB-OVA cross-primes OVA-specific CD8+ CTLs in vivo (23, 34). Additionally, DCs pulsed with intact OVA alone cannot cross-present and cross-prime CTLs (23). For the cross-priming of Ag-specific CTLs by Ag-captured immature DCs, maturation signaling via some surface molecules such as TLR-3 in those DCs is essential (35, 36). Although whole CT up-regulates the expression of MHC class II, B7.1, and B7.2 molecules on DCs in vitro, neither CTA nor CTB alone up-regulates the levels of surface markers on DCs (37, 38). Also, the binding of CTB to GM1 on DCs seems necessary to efficiently take up both CT itself and Ag and to induce cross-presentation by MHC class I molecules on DCs, whereas CTA may not be taken up to affect DCs. When DCs from GM1-lacking mice were matured in vitro, CT failed to up-regulate the expression of maturation markers and, thus, the binding of B subunits in CT to GM1 molecules on DCs is essential for the induction of DC maturation (37). It has been reported that CTA is required to not only assist in maturation but also to generate the migration of DCs (39, 40); therefore, CTB-mediated matured DCs can initiate their migration to secondary lymphoid organs and colocalization with naive T cells (38). Indeed, CT-loaded but not CTA-loaded DCs could migrate from marginal zones to T cell zones in the spleen (39) and from the subepithelial dome region to T cell zones in PPs (40); therefore, both CTA and CTB were essential for cross-priming CTLs in vivo and neither CTA nor CTB alone could induce CTLs at various compartments (Fig. 3). Taken together, although the detailed mechanisms of efficient Ag presentation via MHC class I and the maturation and migration of DCs by CT are still unknown, digested OVA might be efficiently captured by immature gut mucosal DCs in the presence of CTB and the captured Ag may be cross-presented by MHC class I during DC maturation and migration in the presence of CTA, resulting in the induction of mucosal class I MHC molecule-restricted CTLs that may cause the regression of previously established tumors.

OVA-specific CD8+ CTLs were induced among not only the IELs but also the LPLs of the stomach, small intestine, and large intestine by oral administration of OVA plus CT, a higher percentage of OVA-specific CD8 CTLs was observed in the stomach, small intestine, and large intestine in order, and more specific CTLs were always detected among IELs than among LPLs (Fig. 4, E and F). Thus, CTLs are much easier to be induced in the upper and more superficial portions of the gastrointestinal tract when Ags are orally administered with intact CT.

It has been reported that DCs in gastric mucosa are increased in Helicobacter pylori (Hp)-infected mice and that the response of DCs and T cells to Hp Ag is critical for Hp-induced gastritis (41). In the present study, Ag-specific CTLs in the stomach might be generated by mucosally activated DCs in the presence of CT and infiltrate-implanted gastric tumor tissues. It is possible that intestinally activated CTLs might migrate to the tumor-implanted stomach, which might also cause CTL infiltration. Actually, such effector CTLs usually express high levels of αβ2 integrin and can home in to the gastric (42), and small and large intestinal mucosa (43) where mucosal addressin cell-adhesion molecule-1 (MadCAM-1), the ligand of αβ2 integrin, is constitutively expressed by postcapillary endothelial cells in small (44, 45) and large intestinal lamina propria (46). Moreover, the number of gastric αβ2-high T cells increased markedly by oral administration of CT in mice (42). It has also been reported that MadCAM-1 expression is increased in the gastric mucosa after oral administration with cholera vaccine composed of CTB and formalin-inactivated V. cholerae (47); therefore, MadCAM-1 expression in gastric mucosa and the recruitment of effector αβ2high T cells to gastric mucosa might be enhanced by oral administration of the CT adjuvant and, thus, OVA-specific effector CTLs might efficiently infiltrate the OVA Ag-expressing tumor region in the stomach.

In the present study, we found that the growth of dermally implanted tumors was also suppressed by the oral administration of tumor Ag plus whole intact CT. The actual mechanisms for such suppression remains to be elucidated, but there are at least three distinct possibilities: first, the migration of Ag-specific CTLs from the gastrointestinal tract to the skin; second, the migration of Ag-presenting DCs activated in the mucosal compartments by CT; and third, the migration of both cells from the gastrointestinal tract to the skin at the same time. It has been reported that the levels of CCR4 expression, which is associated with T cell homing to the skin, are increased in gastric T cells by infection with Hp in humans (48). Moreover, mucosal DCs that take up Ag might migrate to regional lymph nodes near the dermal tumor and prime the CTLs there, and the CTLs could effectively infiltrate dermal tumor tissue. Indeed, Belyakov et al. demonstrated an opposite mechanism in which skin-derived DCs containing heat-labile enterotoxin of Escherichia coli migrated to PPs and induced mucosal CTLs by transcutaneous immunization of an Ag and CT (49). Although the detailed mechanisms of this migration of DCs between skin and mucosa are unknown, they have clearly shown that DCs can migrate between the mucosa and skin. We are currently comparing the alteration of DCs in the mucosal compartment, spleen, and lymph nodes after oral administration of an Ag plus natural CT.

Unfortunately, such natural CT is not an appropriate mucosal adjuvant for human clinical investigation (50); however, studies
using natural CT would provide important and critical information about the effect of CT that would be useful for mucosal immune activation. Based on the findings obtained by using natural CT in a mouse model system, we could establish much safer protocols with a mutant CT (51) that induces adenosine diphosphate ribosylation and cyclic adenosine monophosphate formation, which may prevent severe diarrhea as well as retain adjuvant properties. Taken together, an artificial CT-based vaccine targeting DCs may provide a strategy for efficient CTL induction and avirulent mucosal cancer vaccination.

Our data also indicate that E.G7-OVA tumor growth was suppressed by OVA-specific CTLs but not NK cells (Fig. 7B). Vaccination with OVA-CT-pulsed DCs protects against E.G7-OVA tumor development in vivo in wild-type, NK-depleted, and CD4-deficient mice but not in CD8-deficient mice (34), indicating that the E.G7-OVA tumor might be controlled by CD8 T cells but not by NK cells or CD4 T cells. In fact, TILs in the suppressed tumor did not show any NK-related cytotoxicity (Fig. 7B). Moreover, it has been demonstrated that in vitro pretreatment of NK cells with CT inhibits NK cell killing of tumor (YAC-1 or P815), because G proteins in NK cell membranes are ADP ribosylated with CT and ribosylation inhibits the lysis of tumor cells (52); therefore, NK cells do not seem to be involved in the suppression of E.G7-OVA growth in vivo.

It has been shown that activated CTLs but not naive CTLs can represent antitumor (22) or antivirus (12) responses in vivo. In the present study, already established E.G7 tumor growth can be suppressed only when OVA-specific CTLs that show specific cytotoxicity without requiring in vitro restimulation are induced, particularly in the mucosal compartment. To our knowledge, this is the first demonstration of the visual suppression of already established tumor growth by the simple oral administration of tumor Ag plus mucosal adjuvant. The findings shown in the present study herald a new era for cancer immunotherapy.

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Disclosures
The authors have no financial conflict of interest.

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