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A Novel Mucosal Vaccine Based on Live Lactococci Expressing E7 Antigen and IL-12 Induces Systemic and Mucosal Immune Responses and Protects Mice against Human Papillomavirus Type 16-Induced Tumors

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Current strategies to prevent or treat human papillomavirus type 16 (HPV-16) infection are promising, but remain costly. More economical but efficient vaccines are thus needed. In this study, we evaluated the protective effects of mucosally coadministered live Lactococcus lactis strains expressing cell wall-anchored E7 Ag and a secreted form of IL-12 to treat HPV-16-induced tumors in a murine model. When challenged with lethal levels of tumor cell line TC-1 expressing E7, immunized mice showed full prevention of TC-1-induced tumors, even after a second challenge, suggesting that this prophylactic immunization can provide long-lasting immunity. Therapeutic immunization with L. lactis recombinant strains, i.e., 7 days after TC-1 injection, induced regression of palpable tumors in treated mice. The antitumor effects of vaccination occurred through a CTL response, which is CD4+ and CD8+ dependent. Furthermore, immunized mice developed an E7-specific mucosal immune response. These preclinical results suggest the feasibility of the low-cost mucosal vaccination and/or immunotherapy strategies against HPV-related cervical cancer in humans. The Journal of Immunology, 2005, 175: 7297–7302.

Several strategies for human papillomavirus type 16 (HPV-16) vaccines have been developed: a prophylactic vaccine based on highly purified virus-like particles was recently used in clinical trials in women, with a significant reduction of the incidence of both HPV-16 infection and HPV-16-related cervical cancer (CxCa), the second cause of cancer-related deaths in women worldwide (1, 2). However, since the vaccine is based on virion capsid proteins (L1 and L2), which are absent in CxCa, it is unlikely that such a vaccine could be used therapeutically in already infected patients. In contrast, the HPV-16 E7 protein is constitutively produced during malignant progression of HPV-16-induced cervical lesions, (3) and could thus be a more effective target for cancer therapy.

Some E7 systemic vaccines have been investigated to elicit an immune response against HPV-16 (3, 4); nevertheless, as most injectable vaccines, costs and the need of trained personal make these new vaccine unsuitable for large vaccination programs, particularly in developing countries. Mucosal vaccines, e.g., administered by oral, intranasal (i.n.), or possibly vaginal route, are more convenient than systemic vaccines, because they are easier to administer and relatively cheap to produce. Moreover, mucosally administered vaccines can stimulate serum-IgG and mucosal-IgA Abs (to neutralize toxins and viruses) and induce CTL activities (5). In addition, because mucosal vaccines are less invasive, mucosal immunizations are more attractive for use in children and immunosuppressed patients.

Strategies to deliver HPV Ags for vaccination via live, attenuated bacterial pathogens are now recognized to pose risks, particularly to immunosuppressed patients. We chose Lactococcus lactis as the Ag delivery vector. This dairy microorganism is present in numerous foods and is noninvasive and nonpathogenic. In the last decade, successful use of L. lactis to codeliver Ags and cytokines to induce a mucosal immune response has been verified (6–13).

We previously engineered two L. lactis (LL) strains: one displays HPV-16 E7 Ag on its cell surface (LL-E7) and the second secretes biologically active IL-12 (LL-IL-12) (9, 10, 13). IL-12 is a potent heterodimeric cytokine that plays an essential role in determining the balance of the immune response: it induces Th1 cells, enhances CTL maturation, promotes NK cell activity, and induces IFN-γ production (14, 15). Moreover, IL-12 possesses adjuvant properties when codelivered with Ags and has been successfully used in cancer immunotherapy. Intranasal administration with these recombinant L. lactis strains induces an E7-specific immune response in mice (9, 10, 13). These encouraging results prompted us to explore whether codelivery of LL-E7 and LL-IL-12 could be used for immunization and immunotherapy of HPV-related CxCa.
Materials and Methods

Recombinant lactococci strains

The construction of recombinant *L. lactis* strains anchoring HPV-16 E7 Ag (LL-E7) and secreting biologically active murine IL-12 (LL-IL-12) has been previously described (9, 10, 13).

Mouse and cell line strains

Specific pathogen-free C57BL/6 mice (females, 6–8 wk of age; Charles River Breeding Laboratories) were housed in a pathogen-free isolator under sterile conditions with water and fed ad libitum in the animal facilities of the Unité d’Écologie et de Physiologie du Système Digestif at the National Institute of Agricultural Research (Jouy-en-Josas, France). All experiments were performed according to protocols in accordance with institutional guidelines.

The mouse (C57BL/6) lung tumor line TC-1 was generated by transduction with a retroviral vector expressing HPV-16 E6/E7 plus a retrovirus expressing activated c-Ha-ras (16). TC-1 cells were grown in RPMI 1640 supplemented with 10% FCS, 50 U/ml penicillin, 50 U/ml streptomycin, and 0.4 mg/ml G418.

Preparation of live bacterial inocula for immunization

*Lactococcus lactis* was grown in M17 (Difco) supplemented with 1% glucose at 30°C without shaking. To induce the nisin promoter, strains were grown until OD<sub>600</sub> = 0.6, followed by induction with 10 ng/ml nisin (Sigma-Aldrich) for 1 h as previously described (17). Cellular pellets were then harvested by centrifugation at 3000 × g at 4°C and washed three times with sterile PBS. The pellet was suspended in PBS to a final concentration of 1 × 10<sup>9</sup> CFU. Plate counts were performed with all inocula to corroborate the CFU administered. IL-12 and E7 production were controlled by immunoblotting before inoculation. Abs used were mouse anti-IL-12 (R&D Systems) and anti-E7 (Santa Cruz Biotechnology).

Prophylactic protocol for an HPV-16 tumor model in mice

Groups of mice were immunized intranasally (i.n.) with 1 × 10<sup>8</sup> CFU from each induced recombinant lactococci strain (LL-E7 and LL-IL-12 suspended in 10 μl of PBS and 5 μl was administered with a micropipette into each nostril) on days 0, 14, and 28. Control mice received either PBS or identical quantities of an isogenic strain of *L. lactis* which does not produce any recombinant protein (LL). Seven days after the last administration (day 35), the mice were challenged by s.c. injection in the right rear flank with 5 × 10<sup>5</sup> TC-1 tumor cells in 100 μl of PBS. The dimensions of the tumor at the site of injection of TC-1 cells were measured every week in two perpendicular directions with a caliper, and the volume of the tumor was estimated as: (length × width<sup>2</sup>) / 2.

CTL response against TC-1 tumor cells

Vaccinated mice were sacrificed 7 days after the last injection (day 35) and CTL activity was determined using a nonradioactive cytotoxicity assay kit (Roche Applied Science) based on the measurement of lactate dehydrogenase (LDH) activity released by lysed cells. Briefly, spleen cells were separated on a Lympholyte-M (Cedarlane Laboratories) density gradient, and 1 × 10<sup>6</sup> splenocytes were cocultured with 1 × 10<sup>6</sup> mitomycin C-treated TC-1 cells (100 μg/ml) and a combination of two E7 peptides: E7<sub>30-57</sub>, which contains the MHC class I epitope (18) and specifically stimulates CD8<sup>+</sup> cells, and E7<sub>49-67</sub>, which contains the MHC class II epitope (19) and specifically stimulates CD4<sup>+</sup> cells (10 μg/ml each one) in RPMI 1640 supplemented with 1% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. Five days after stimulation, splenocytes were recovered and used as effector cells, and 1 × 10<sup>6</sup> viable TC-1 cells were used as target cells at a ratio of 1:1, 10:1, and 30:1 (tested in duplicate). After 5 h of culture, supernatants were recovered and LDH activity was determined. To characterize the roles of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in E7-induced cytotoxicity, T cell subset depletion was performed with anti-CD4 mAb GK1.5 (R&D Systems) for CD<sup>+</sup> or anti-CD8 mAb 53-6.7 (R&D Systems), mAbs were mixed with effector cells at various E:T ratios, respectively, before being added to target cells in a final concentration of 10 μg/ml and incubated for 45 min at 4°C to block CD4<sup>+</sup> or CD8<sup>+</sup> T cells. The mean percentage of specific lysis of triplicate wells was: cytotoxicity (%) = [(experimental value – spontaneous LDH release)/(maximum LDH release – spontaneous LDH release)] × 100.

Measurement of E7-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells

Splenocytes from vaccinated mice were incubated with E7<sub>49-57</sub> peptide (MHC class I epitope) or E7<sub>30-57</sub> peptide (MHC class II epitope) and the number of E7-specific IFN-γ-producing T cells was measured by ELISPOT (mouse IFN-γ; R&D Systems). One week after the last immunization, mice were sacrificed and splenocytes were isolated as described above. Single-cell suspension of splenocytes (1 × 10<sup>6</sup> cells/well) were stimulated overnight with 10 μg/ml MHC class I or MHC class II epitope and 0.5 ng/ml rIL-2 (R&D Systems) in a 24-well plate in 1 ml of RPMI 1640 supplemented with 10% FCS at 37°C under 5% CO<sub>2</sub>. After 24 h, cells were harvested, washed, and plated at a concentration of 2.5 × 10<sup>5</sup> cells/well in a 96-well polystyrene plate coated with monoclonal-specific mouse IFN-γ Ab. Plates were incubated for 4 h at 37°C in 5% CO<sub>2</sub> and then washed four times. One hundred microliters of detection Ab was then added and incubated overnight at 4°C. The next day plates were washed four times and 100 μl of streptavidin-alkaline phosphatase was added. Incubation was pursued for 2 h at room temperature and then plates were washed four times. The development of the reaction was performed using 0.1% of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate for 1 h. The reaction was stopped by the addition of deionized water and after removal of water excess the plates were allowed to dry before counting the individual spots with a dissecting scope.

Immunotherapy protocol for in vivo tumor regression experiments

For therapeutic experiments, the mice were first challenged s.c. in the right rear flank with 5 × 10<sup>5</sup> TC-1 tumor cells, followed by three administrations of recombinant live lactococci (at intervals of 7 days) 7, 14, or 18 days later. At day 7, 100% of the mice challenged had developed a palpable tumor.

Statistical analyses

All data are expressed as mean values and SDs. The effect of LL-E7 and LL-E7/IL-2 treatments on tumor appearance (prophylaxis) or tumor persistence (therapy) was assessed by comparing the distribution of tumor-bearing and tumor-free animals among groups using a χ<sup>2</sup> analysis. The effect of LL-E7 and LL-E7/IL-12 on the tumor volume of tumor-bearing animals and CTL response was then analyzed using a one-way-ANOVA; when significant, the Bonferroni/Dunn multiple comparison test was applied to determine the specific differences between means. For parameters for which variances were unequal, i.e., T cells in ELISPOT assay, the effect of LL-E7 and LL-E7/IL-12 was analyzed using the nonparametric Kruskal-Wallis test. Statistical significance was set at p < 0.05. Calculations were performed using Statview software (version 5.0; SAS Institute).

Results

Intranasal vaccination with LL-E7 and LL-IL-12 generates long-term immunity against a HPV-16 tumor model in mice

To determine whether i.n. coadministration with LL-E7 and LL-IL-12 leads to antitumor effects, we performed in vivo tumor protection experiments using an HPV-16 tumor model in which injection of the TC-1 cell line provokes lethal tumors (16). Groups of mice were i.n. immunized three times (at intervals of 2 wk, see Materials and Methods) with LL-E7 alone or in combination with LL-IL-12. Seven days after the last immunization, the mice were challenged s.c. with 5 × 10<sup>5</sup> TC-1 and monitored once a week. As negative control, either PBS or an isogenic strain of *L. lactis* (LL) that produces neither E7 nor IL-12 was administered to a group of mice. Because the group of unvaccinated PBS mice developed aggressive tumors similar to mice immunized with LL (data not shown), LL administration was chosen as negative control in subsequent experiments. As shown in Fig. 1, 100% of mice vaccinated with LL alone developed aggressive tumors which killed them within 35 days, with a median tumor size of ~6 cm<sup>3</sup> (Fig. 1). In contrast, 35% of mice vaccinated with LL-E7 alone remained tumor free over the test period (~100 days) and in the remaining 65% tumor-bearing mice, the median size (~3 cm<sup>3</sup>; p < 0.001; Fig. 1A) was 2-fold lower than the one measured in LL-immunized mice. Covaccination with LL-E7 and LL-IL-12 gave the best response, as 50% of mice remained tumor free ~100 days after TC-1 challenge and the median size in the remaining 50% tumor-bearing animals (~1 cm<sup>3</sup>; Fig. 1A) was significantly reduced (p < 0.001 and p < 0.0001) when compared with those of LL- and LL-E7-treated mice.
Antitumoral activity elicited by covaccination with LL-E7 and LL-IL-12 appeared to be long-lasting as 10 tumor-free animals were rechallenged 3 mo later with TC-1 cells in the flank opposite that of the first inoculation and remained tumor free for up to 6 mo (Fig. 1B, arrow). Tumor-free mice appeared to be healthy at the end of the experiment and no weight loss was observed. In addition, no visible toxicity was detected after careful examination of mice during autopsy. Altogether, these findings suggest that coadministration of LL-E7 and LL-IL-12 induces antitumoral effects against a HPV-16 tumor model in mice.

Vaccination with LL-E7 and LL-IL-12 enhances the E7-specific CTL response

To elucidate the mechanism of protection against TC-1 tumors induced by recombinant lactococci, we first determined whether a CTL response was induced in mice vaccinated with LL-E7 and LL-IL-12. Splenocytes were isolated from immunized mice 7 days after the last administration of recombinant lactococci and stimulated in vitro with mitomycin C-treated TC-1 cells and both E7 peptides (E7<sub>49–57</sub> and E7<sub>30–67</sub>). Stimulated splenocytes were then tested for recognition and lysis of fresh viable TC-1 cells. As shown in Fig. 2, spleen cells from mice vaccinated with LL-E7/LL-IL-12 had significant CTL activity (p < 0.05) to lyse target cells. In contrast, mice vaccinated with LL-E7 alone or LL showed very weak CTL induction (Fig. 2). This finding suggests that mucosal covaccination with live LL-E7 and LL-IL-12 induces E7-specific CTL cells, which is likely responsible for tumor protection.

Stimulation of CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses by vaccination with LL-E7 and LL-IL-12

Because CD8<sup>+</sup> T cells have a recognized role in antitumor immunity, we then evaluated the CD8<sup>+</sup> T cell-dependent immune response. For this, splenocytes from animals vaccinated with LL-E7 and LL-IL-12 were stimulated in vitro with E7<sub>49–57</sub> peptide, and IFN-γ production was then analyzed at the single-cell level using the ELISPOT assay. Coadministration of LL-E7 and LL-IL-12 generated higher numbers of E7-specific IFN-γ-secreting CD8<sup>+</sup> T cells (p < 0.05, 210 ± 30/3 × 10<sup>3</sup> splenocytes) than administration of LL-E7 alone (90 ± 25/3 × 10<sup>3</sup> splenocytes) or LL alone (2 ± 1/3 × 10<sup>3</sup> splenocytes) (Fig. 3). These data indicate that LL-IL-12 is able to enhance E7-specific CD8<sup>+</sup> T cell responses when coadministered with LL-E7. This is consistent with our previous observation that the greatest stimulation of CD8<sup>+</sup> T cells was detected in mice vaccinated with the combination of LL-E7 and LL-IL-12 (9).

We also evaluated the CD4<sup>+</sup> T cell-dependent immune response generated by vaccination with LL-E7 and LL-IL-12. Splenocytes from vaccinated mice were stimulated in vitro with E7<sub>30–67</sub> and analyzed by ELISPOT. As shown in Fig. 3, IFN-γ levels were enhanced by coadministration of LL-E7 and LL-IL-12 (p < 0.05, 260 ± 20/3 × 10<sup>3</sup> splenocytes) compared with administration of LL-E7 (40 ± 25/3 × 10<sup>3</sup> splenocytes) or of LL alone (1 ± 0.5/3 × 10<sup>3</sup> splenocytes). These data show that coadministration with LL-E7 and LL-IL-12 induces E7-specific CD4<sup>+</sup> T cell responses.
Both CD8⁺ and CD4⁺ T cells are essential for the generation of antitumor response elicited by coadministration of LL-E7 and LL-IL-12.

To understand the relative roles of T cell subsets in tumor protection induced by coadministration of LL-E7 and LL-IL-12, CD4⁺ and CD8⁺ T cells were blocked with specific mAbs, and the in vitro CTL response was assayed. As shown in Fig. 4, CTL activity elicited by LL-E7 and LL-IL-12 appeared to be specific to both CD4⁺ and CD8⁺, as blocking with the respective specific mAbs led to a significant loss of specific lysis (p < 0.05). Taken together, these data support the roles of both CD4⁺ and CD8⁺ T cells in the induction of tumor protection upon vaccination with LL-E7 and LL-IL-12.

Coadministration with LL-E7 and LL-IL-12 causes regression of established tumors.

To assess the therapeutic effects of coadministration of LL-E7 and LL-IL-12, mice were first challenged with the TC-1 tumor cell line before starting the immunotherapy protocol. Once 100% of the mice had formed a palpable tumor (day 7), the immunotherapy was started. As shown in Fig. 5A, only LL-E7/IL-12 treatment results in total tumor regression in 35% of immunized animals and remained tumor free over the test period (100 days). Moreover, tumor growth was diminished in the remaining 65% of mice when compared with LL and LL-E7 treatments (Fig. 5). In contrast, no tumor regression was observed in mice treated with LL (which had rapidly growing aggressive tumors with ulcerated surfaces) or LL-E7 alone. Note that the median size of the tumors (~3.5 cm³) is lower in LL-E7-treated mice than in LL-treated mice. Although LL-E7 treatment did not induce total tumor regression, some decrease was observed when compared with the LL-treated group.

Discussion

Of the ~100 million women who have already been infected with HPV, some 5 million are susceptible to persistent infections that could give rise to CxCa if left untreated (20). The widespread occurrence of HPV calls for measures not only to prevent new...
infections, but also to provide treatment for already infected individuals. Vaccines developed to date have proven effective in prophylaxis (2), but because of the absence of virion capsid proteins in CxCa, are not expected to have therapeutic effects. Both prophylactic and therapeutic vaccine regimens developed here led to effective prevention and/or regression of HPV-induced tumors in the murine model.

The use of adjuvants has been shown to be essential in the conception of E7-based therapeutic vaccines designed to reduce or abolish tumor growth (21). Cytokine IL-12 was shown to have a marked antitumor effect on different tumors, as an effector of both innate and adaptive immunity (for review, see Ref. 22). However, systemic IL-12 therapies have been limited by high levels of toxicity (23). The live mucosal L. lactis-based vaccine we developed to prevent or treat CxCa delivers surface-anchored E7 Ag and secreted IL-12. The use of lactococci limits risks of toxicity associated with IL-12, since the passage (24 h) of noncolonizing L. lactis ensures only transient cytokine expression. Additionally, i.n. administration, rather than a systemic one, may explain why no toxicity in mice was observed (24).

Mucosal vaccination with recombinant L. lactis strains evoked a strong E7-specific cellular immune response, which likely contributed to the anti-HPV-16 tumor effects observed after prophylactic and therapeutic treatments. Indeed, we observed that in vitro stimulation with E7 

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749-570
\end{equation}

or E7 

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30-670
\end{equation}

peptides induced IFN-γ production by CD8+ or CD4+ T cells, respectively, in immunized mice. E7 

\begin{equation}
49-570
\end{equation}

(RAHNVNTF) corresponds to the HPV-16 E7 H2-D8-restricted CTL epitope (18) and E7 

\begin{equation}
30-670
\end{equation}

(DSSEEEDEIDGPAAQGAE) stimulates. However, in preliminary experiments we reported to stimulate CD4+ T cells but not CD8+ T cells are responsible for IFN-γ production by E7 

\begin{equation}
30-670
\end{equation}

stimulation. However, in preliminary experiments we compared stimulation with complete E7 protein (previously reported to stimulate CD4+ T cells; Refs. 25 and 26) and E7 

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30-670
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peptide CD4+ T cells, and we observed that in both models, CD4+ T cells but not CD8+ T cells are responsible for IFN-γ production. These experiments were only performed on sacrificed mice 7 days after the last injection and not on long-term protected mice. We hypothesize that this long-lasting protection is due to the same CTL response as determined in Fig. 2.

Interestingly, our vaccine also induced both systemic and mucosal humoral responses. We previously showed that i.n. administration of LL-E7 induces a humoral immune response in mice as corroborated by Ab production (10). In this work, we also evaluated whether mice vaccinated with recombinant lactococci are able to induce mucosal E7-specific IgG and IgA Abs in bronchoalveolar lavage fluids. The results revealed that mice immunized with LL-E7/LL-IL-12 produced higher E7-specific IgG and IgA Abs than mice vaccinated with LL-E7 or LL alone (data not shown). These data suggest that i.n. vaccination with live LL-E7 induces an Ag-specific mucosal immune response and that coadministration with LL-IL-12 enhances this response.

This study demonstrates the feasibility of developing live, recombinant nonpathogenic and noninvasive L. lactis-based vaccines to help control HPV-related CxCa by prophylaxis and treatment. Vaccination by mucosal, rather than systemic, delivery offers several advantages: 1) easy administration without the need for trained personnel, 2) reduction of secondary effects, and 3) reduced hygienic risk associated with the use of syringes. These advantages can considerably reduce costs and increase safety of vaccination campaigns, mainly in developing countries. In conclusion, this work is the first demonstration of a therapeutic effect against cancer (i.e., CxCa) induced by mucosal administration of live, recombinant lactococci. It further emphasizes the potential of recombinant lactococci for cancer immunotherapy. Moreover, biological containment strategy is now available to use genetically engineered L. lactis secreting IL-10 in experimental therapy for Crohn’s disease patients as recently approved by Dutch authorities (Ref. 27 and L. Steidler, personal communication).

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Disclosures

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