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An inducible surface presentation system improves cellular immunity against human papillomavirus type 16 E7 antigen in mice after nasal administration with recombinant lactococci

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Human papillomavirus type 16 (HPV-16) is the major causative agent of cervical cancer. To date, vaccine strategies against HPV-16 are based on the ability of the E7 oncoprotein to elicit an immune response against this virus. In this study, the use of an inducible or a constitutive system to produce the HPV-16 E7 protein in *Lactococcus lactis*, a non-pathogenic and non-invasive Gram-positive bacterium, was compared. The highest E7 production was obtained with the inducible system. When mice were immunized intranasally with recombinant lactococci expressing either inducible or constitutive E7, an antigen-specific cellular response (i.e. secretion of IL2 and IFN-γ cytokines) was evoked and was substantially higher in mice receiving *L. lactis* expressing E7 with the inducible system. As bacterial antigen location may influence the immune response, recombinant *L. lactis* strains that produced E7 in three cellular locations, intracellular, secreted or cell-wall-anchored were evaluated. The highest immune response was elicited by administration of *L. lactis* producing an inducible cell-wall-anchored form of E7 protein. These promising results represent a step towards the development of a new, safe mucosal vector to treat HPV-related cervical cancer.

INTRODUCTION

Epidemiological data have clearly shown that human papillomavirus type 16 (HPV-16) infection is the main aetiological factor for cervical cancer (CxCa) (Furumoto & Irahara, 2002). Worldwide, ~400 000 women die annually from CxCa (Parkin *et al.*, 1999). A prophylactic and/or therapeutic vaccine against this virus is thus a priority to prevent or to treat, respectively, CxCa. A prophylactic vaccine based on highly purified virus-like particles has recently been successfully used in trials in women, with a significant reduction observed in the incidence of both HPV-16 infection and related CxCa (Koutsky *et al.*, 2002). However, such vaccines could probably not be used therapeutically in already-infected patients because the virion capsid proteins are not detected in CxCa. The HPV-16 E7 protein, constitutively produced in cervical carcinomas, is required for the transformation process (Baker *et al.*, 1987; Bedell *et al.*, 1987; Dyson *et al.*, 1989; Tanaka *et al.*, 1989) and is considered a good antigen candidate for the development of a therapeutic vaccine against CxCa.

Several studies have investigated the use of bacteria as E7 antigen delivery vehicles to elicit an immune response against HPV-16. In these studies, the vectors used were attenuated strains of pathogenic bacteria such as *Salmonella* and *Mycobacterium* spp. (Londoño *et al.*, 1996; Jabbar *et al.*, 2000). Although these recombinant strains elicited immune responses, invasiveness of the vectors and risks of reversion to pathogenicity limit their use in vulnerable groups such as immunocompromised patients or children. There is thus a need for the development of a new generation of safe delivery vehicles.

Lactic acid bacteria (LAB) are promising candidates as safe vehicles for *in vivo* delivery of antigens. Compared with attenuated bacterial vectors, LAB are non-pathogenic and non-invasive Gram-positive organisms and 'generally recognized as safe' (GRAS). Furthermore, some LAB species

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Abbreviations: CxCa, cervical cancer; HPV-16, human papillomavirus type 16; LAB, lactic acid bacteria.

reportedly exert probiotic effects in humans and some are widely used in the food industry. Our team previously reported E7 production in *Lactococcus lactis*, the model LAB (Bermúdez-Humarán *et al.*, 2002, 2003a,b; Cortes-Perez *et al.*, 2003). Vaccination through mucosal routes using *L. lactis* constitutes an easy and low-cost administration method. In addition, as *L. lactis* is a non-commensal and transient bacterium in the digestive tract (Drouault *et al.*, 1999; Geoffroy *et al.*, 2000), the risk of eliciting a tolerance response to the antigen delivered is diminished compared with persistent bacteria.

Although high production of heterologous proteins in *L. lactis* has been obtained using constitutive promoters (de Vos, 1999), continuous high-level production of a protein could lead to intracellular accumulation or degradation in the cytoplasm, which could, in some cases, be deleterious to the cell. Thus, in this study, we evaluated the use of a constitutive system versus an inducible system [nisin inducible system, NICE (de Ruyter *et al.*, 1996; Kuipers *et al.*, 1998)] to produce the HPV-16 E7 protein in *L. lactis*. NICE is a versatile system where gene expression can be up-regulated more than 1000-fold (de Ruyter *et al.*, 1996). Furthermore, as immunogenicity may depend on the antigen location, the immune response was evaluated for three recombinant *L. lactis* strains targeting E7 antigen to the cytoplasm, the medium or to the cell-wall.

The highest production of E7 was obtained with the inducible system. When mice were immunized with *L. lactis* targeting E7 to different cellular locations, an E7-specific cellular response (i.e. secretion of IL2 and IFN-γ cytokines) was evoked and was higher in mice receiving *L. lactis* producing an inducible cell-wall-anchored form of E7. These strains are thus good candidates for the therapy and prevention of HPV-related CxCa.

METHODS

Bacterial strains, plasmids and DNA manipulations. Plasmid constructs used in this study allowing the inducible production of HPV-16 E7 protein in the cytoplasm (pCYT : E7) or secreted into the culture medium (pSEC : E7) in *L. lactis*: NZ(pCYT : E7) and NZ(pSEC : E7) have been described previously (Bermúdez-Humarán *et al.*, 2002). *L. lactis* was grown in M17 (Difco) supplemented with 1 % glucose (GM17) at 30 °C without shaking. Plasmid constructions were established in *L. lactis* as described previously (Langella *et al.*, 1993) and maintained by the addition of 10 μg chloramphenicol ml^{-1} or 5 μg erythromycin ml^{-1}. DNA manipulations were performed using standard methods essentially as described by Sambrook *et al.* (1989).

Plasmid construction to express E7 protein in *L. lactis* constitutively. To express E7 protein in *L. lactis* under the transcriptional control of a constitutive promoter, the following DNA manipulations were performed: briefly, an E7 : trpA cassette was purified from pCYT : E7 digested with *Bam*HI-Klenow/*Spe*I and cloned into a pIL backbone purified from pVE5546 digested with *Nru*I/*Spe*I (Dieye *et al.*, 2001), resulting in pILCYT : E7. In this plasmid, the E7 gene is under the control of P_{59}, a lactococcal constitutive promoter (van der Vossen *et al.*, 1987) commonly used for heterologous gene expression in *L. lactis* (Piard *et al.*, 1997; Chatel *et al.*, 2001). This plasmid, designed for constitutive cytoplasmic E7 production, was introduced into *L. lactis* MG1363 (Gasson, 1983) resulting in MG(pILCYT : E7).

Plasmid constructions to express a cell-wall-anchored E7 protein in *L. lactis* with an inducible system. Recently, we have described a pILCWA : E7 vector to display E7 protein at the cell wall of *L. lactis* (Cortes-Perez *et al.*, 2003). However, pILCWA : E7 is a derivative of pVE5547, a large theta-replicating plasmid that is difficult to manipulate (Dieye *et al.*, 2001). An SP_{Usp}-E7-CWA_{M6} cassette was transferred from pILCWA : E7 into a pGK derivative, a smaller *Escherichia coli*–Gram-positive shuttle vector that is easier to manipulate (Kok *et al.*, 1984; Bermúdez-Humarán *et al.*, 2002, 2003c). Briefly, the SP_{Usp}-E7-CWA_{M6} cassette was purified from pILCWA : E7 digested with *Bgl*II/*Spe*I and cloned into the pGK backbone purified from pSEC : E7 digested with *Bgl*II/*Spe*I. The resulting plasmid, pCWA : E7, was introduced into *L. lactis* NZ9000 resulting in NZ(pCWA : E7).

Immunoblotting. Fresh medium was inoculated 1 : 50 (v/v) with an overnight culture and incubated at 30 °C without shaking. To induce the nisin-inducible promoter, strains were grown until OD_{600} ~0·6, followed by induction with 10 ng nisin ml^{-1} (Sigma) for 1 h. For the *L. lactis* strain expressing E7 protein under the control of P_{59} [MG(pILCYT : E7)], cultures were harvested at OD_{600} ~0·8, which corresponds to an OD_{600} similar to that reached after 1 h of nisin induction for other strains. Sample preparation and immunoblotting assays were performed as described before (Bermúdez-Humarán *et al.*, 2002, 2003a), using anti-E7 antibodies for immunodetection (HPV-16 E7; Santa Cruz Biotechnology).

Preparation of live bacterial inoculum. Bacterial cultures were prepared as described above. At OD_{600} ~0·8 for both constitutive and nisin-induced strains, cell pellets were harvested by centrifugation at 3000 ***g*** at 4 °C and washed three times with sterile PBS. The pellets were suspended in PBS to a final concentration of 1×10^9 c.f.u. Plate counts were performed to check the number of c.f.u. administered and E7 production was assessed by immunodetection.

Immunizations. Groups of five C57BL/6 mice (6–8 weeks; Jackson Laboratory, Bar Harbor, ME) were immunized intranasally with 1×10^9 c.f.u. of each induced recombinant *L. lactis* strain (suspended in 10 μl PBS; 5 μl was administered with a micropipette into each nostril) on days 0, 14 and 28. Mice were partially anaesthetized by intraperitoneal injection of ketamine (1·5 mg for 100 g of weight; Cheminova de México). Control mice received identical quantities of wild-type (wt) *L. lactis* or PBS alone. Experiments were performed according to protocols approved by the International Animal Studies Committee.

Determination of IL2 and IFN-γ cytokine production in splenocytes. Mice immunized with recombinant *L. lactis* strains and control mice were sacrificed on day 35. Splenocytes were separated on a Ficoll-Hypaque (Sigma) density gradient. A total of 2×10^6 cells ml^{-1} in AIM-V medium (Gibco) were plated in a 24-well plate (2 ml per well), at 37 °C under 5 % CO_2. Splenocyte suspensions were restimulated with 2 μg of a synthetic E7 peptide (RAHYNIVTF) to determine whether *in vitro* restimulation induced a peptide-specific cellular response. After 24 h, cell suspensions were filtered and supernatants were examined for the presence of IL2 and IFN-γ cytokines by ELISA (R&D Systems).

Statistics. Student's *t*-test was performed using the MINITAB computer software package.

RESULTS AND DISCUSSION

Evaluation of mucosal immune response in mice after intranasal administration of *L. lactis* producing E7 antigen under the transcriptional control of either a constitutive or an inducible promoter

In the last decade, several studies have used *L. lactis* as an antigen delivery vehicle to develop safe, live vaccines (Robinson *et al.*, 1997; Steidler *et al.*, 1998; Chatel *et al.*, 2001; Enouf *et al.*, 2001; Bermúdez-Humarán *et al.*, 2002; Ribeiro *et al.*, 2002; Xin *et al.*, 2003). In these studies, different transcriptional and translational lactococcal signals have been used to express antigens. However, no report correlates the efficacy of expression systems and gene expression control by a constitutive or an inducible promoter. To address this question, E7 production was analysed in MG(pILCYT : E7) and NZ(pCYT : E7) by immunoblot experiments using anti-E7 antibodies (Fig. 1a). In the cell fraction of NZ(pCYT : E7), one band was detected at the expected size for native E7 (19 kDa) (Bermúdez-Humarán *et al.*, 2002). A similar band was detected in the cell fraction of MG(pILCYT : E7); however, the intensity of the E7 signal was about threefold lower than NZ(pCYT : E7). As expected, no E7 signal was detected in the supernatant samples of either strain. This result shows that, under these induction conditions, NZ(pCYT : E7) produces a larger amount of E7 than does MG(pILCYT : E7). The ability of these two strains to induce an immune response in mice was compared. Groups of five mice were immunized with MG(pILCYT : E7) or induced NZ(pCYT : E7). Seven days after the last boosting (day 35), mice were sacrificed and levels of IFN-γ and IL2 produced by cytotoxic T lymphocytes (CTL) were measured by ELISA, in samples prepared from splenocytes restimulated *in vitro* with an HPV-16 E7-specific CTL epitope (RAHYNIVTF; Fig. 1b and c). Splenocytes prepared from immunized mice produced levels of IL2 (Fig. 1b) and IFN-γ (Fig. 1c) that were significantly higher than the controls (i.e. splenocytes of non-immunized mice or mice immunized with wt *L. lactis*). Both IL2 and IFN-γ levels were approximately twofold higher in mice immunized with NZ(pCYT : E7) than in mice immunized with MG(pILCYT : E7) (Fig. 1b and c). These results show (i) that both recombinant strains are able to induce an antigen-specific CTL response in mice and (ii) that E7 immune response is correlated to the dose of antigen delivered by recombinant lactococci. The amount of E7 delivered by NZ(pCYT : E7) induced culture was indeed approximately threefold higher

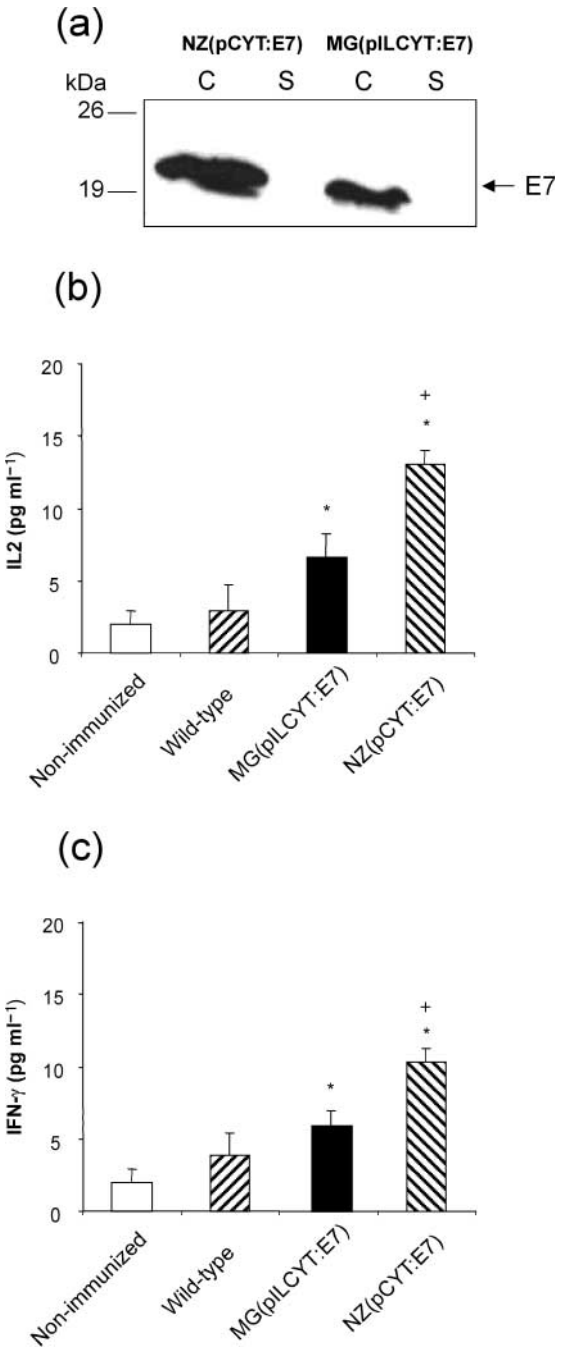

Fig. 1. Evaluation of the capacity of *L. lactis* to produce E7 antigen under transcriptional control of either a constitutive or an inducible promoter. (a) E7 production was analysed by Western blot of protein extracts of strains NZ(pCYT : E7) (inducible promoter) and MG(pILCYT : E7) (constitutive promoter). C, Cell lysate; S, supernatant fraction. Positions and sizes of molecular mass markers are indicated on the left. (b and c) Evaluation of immune response in mice immunized with the two recombinant *L. lactis* strains producing E7 under either a constitutive or an inducible promoter. Levels of IL2 (b) and IFN-γ (c) were assayed by ELISA following immunization of mice with 1×10^9 c.f.u. of different strains of *L. lactis*: wt, MG(pILCYT : E7) and NZ(pCYT : E7). A control placebo group of non-immunized mice was included. Values were recorded as the mean and standard deviation of five mice per treatment group. Statistically significant differences ($P < 0{\cdot}05$) are denoted by an asterisk (*) between controls and immunized groups or by a cross (+) between NZ(pCYT : E7) and MG(pILCYT : E7) immunized groups. These data are representative of three separate experiments showing similar results.

than that delivered by MG(pILCYT : E7) culture (Fig. 1a). These results led us to use inducible strains such as NZ(pCYT : E7) for further experiments.

Comparison of immune response in mice after intranasal administration of recombinant *L. lactis* targeting E7 to different cellular localizations

As protein localization may influence immunogenicity (Norton *et al.*, 1996; Reveneau *et al.*, 2002), we analysed the immune response elicited in mice by three different strains of *L. lactis* producing the E7 antigen either in the cytoplasm, secreted into the external medium or cell-wall-anchored, using strains NZ(pCYT : E7), NZ(pSEC : E7) and NZ(pCWA : E7), respectively. Our hypothesis was that an exported protein (secreted into the medium or cell-wall-anchored) would have direct contact with the target (i.e. the immune system). Protein export may thus be a better strategy compared with intracellular production, which requires cellular lysis for protein delivery. Before immunizations, E7 production in the different induced cultures was analysed by Western blotting and immunodetection using anti-E7 antibodies. As previously observed in Fig. 1(a), samples of induced NZ(pCYT : E7) cultures contained a band that corresponded to native E7 in the cell fraction (Fig. 2a). No E7 signal was detected in the supernatant. For NZ(pSEC : E7), a very faint band corresponding to SP_{Usp}-E7 precursor (preE7) was observed in the cell fraction, whereas an intense band corresponding to secreted mature E7 was detected in the supernatant fraction (Fig. 2a). As previously reported (Bermúdez-Humarán *et al.*, 2002), E7 is very efficiently secreted (~95 % of the over-expressed protein is found in the supernatant) and the yield is about three- to fivefold higher than that obtained with the intracellular form (Fig. 2a). For NZ(pCWA : E7), one major band was detected in the cell fraction at the expected size (~38 kDa) for an SP_{Usp}-E7-CWA_{M6} precursor (preCWA-E7) together with two other bands of lower molecular mass corresponding to the E7-CWA_{M6} form, which results from the cleavage of SP_{Usp}, and the mature E7-CWA generated after processing of CWA_{M6} (i.e. cleavage of CWA_{M6} and covalent link between E7 and the cell wall). A third faint band was also detected between the preCWA-E7 and E7-CWA_{M6} forms, which probably corresponds to an alternative cleavage product of preCWA-E7. Confirmation of E7 display at the cell surface of *L. lactis* was performed by immunofluorescence, essentially as described before (Fig. 2b; Cortes-Perez *et al.*, 2003). These results showed that, even though induction levels were similar, the amount of E7 produced in NZ(pCYT : E7) was significantly (three- to fivefold) lower

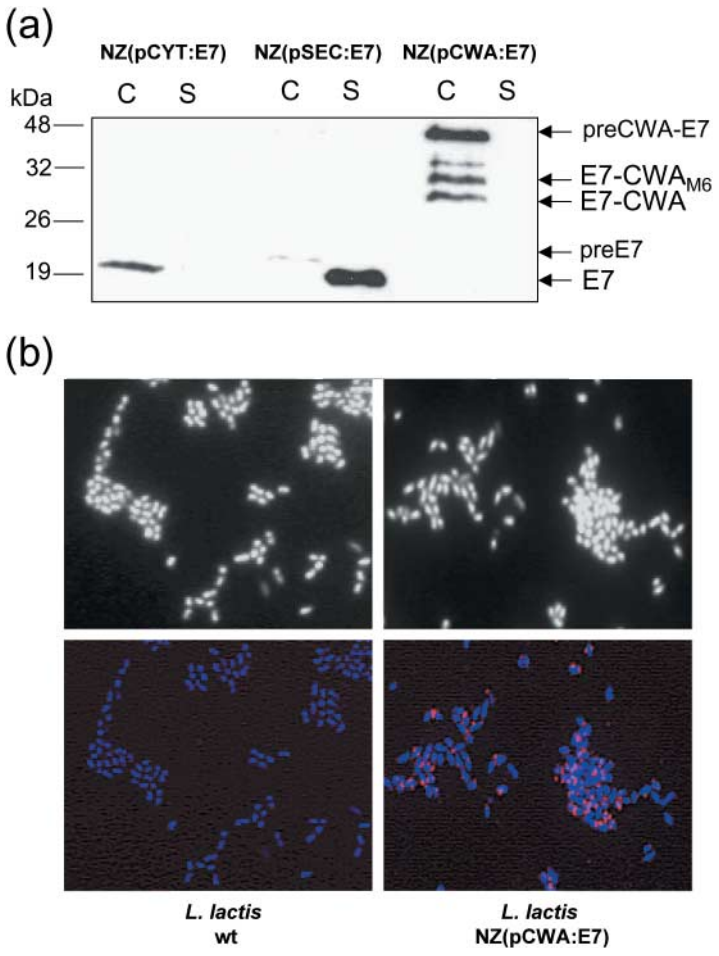

Fig. 2. Analysis of recombinant *L. lactis* strains producing E7 in three different cellular localizations and detection of E7 at the cell-wall surface by immunofluorescence. (a) E7 production was analysed by Western blot of protein extracts of strains NZ(pCYT : E7) (intracellular E7 production), NZ(pSEC : E7) (secreted E7 production) or NZ(pCWA : E7) (cell-wall-anchored E7 production). C, Cell lysate; S, supernatant fraction. Positions and sizes of molecular mass markers are indicated on the left. The third faint band between preE7 and E7-CWA_{M6} forms probably corresponds to an alternative cleavage product of preE7. (b) Recombinant NZ(pCWA : E7) or wt *L. lactis* samples were treated with specific anti-E7 mAbs and then fluorescence-stained with goat anti-IgG conjugate Alexa Fluor 546 dye (lower panels). Corresponding non-stained microscopic fields (DAPI stained) are shown (upper panels). Magnification, ×1000.

than that of NZ(pSEC : E7) or NZ(pCWA : E7). However, in NZ(pCWA : E7), most of the E7 is found in a precursor (intracellular) form. The amount of E7-CWA displayed at the cell surface was similar to that produced in NZ(pCYT : E7), and was estimated to be $\sim$2 μg ml^{-1} by comparison with the signals of known amounts of E7 by quantitative scanning of blots after immunodetection (Image-Quant; Bermúdez-Humarán *et al.*, 2002).

Immunogenicity of the E7 antigen produced in the three different localizations by *L. lactis* strains was then tested. Groups of five mice were immunized intranasally and levels of IL2 and IFN-γ cytokines were measured as described above. Splenocytes obtained from mice immunized with NZ(pCWA : E7) and restimulated *in vitro* produced higher cytokine levels than those obtained from mice immunized with the NZ(pCYT : E7) strain (Fig. 3a and b). As discussed above, in NZ(pCWA : E7), the amount of cell-wall-anchored E7 was estimated to be $\sim$2 μg ml^{-1}, similar to the cytoplasmic E7 production in NZ(pCYT : E7). The higher immunogenicity of E7 delivered by NZ(pCWA : E7) could result from the cell surface display of E7 in *L. lactis*. This type of result has been previously reported in LAB (Norton *et al.*, 1996; Reveneau *et al.*, 2002), and was attributed either to a better accessibility to the immune system when the antigen is exposed at the bacterial surface or to some adjuvant properties of the LAB vector itself. Although the amounts of E7 in NZ(pCYT : E7) and surface-exposed E7-CWA in NZ(pCWA : E7) are quite similar, the total amounts of E7 forms are much higher in NZ(pCWA : E7) than in NZ(pCYT : E7). The greater immune response obtained with NZ(pCWA : E7) could thus be due to a combination of cell surface display and a dose-dependent response (after release of the preCWA-E7 by cell lysis), as shown above. The immune response obtained with NZ(pSEC : E7) was lower than that observed in mice immunized with NZ(pCYT : E7) and NZ(pCWA : E7) (Fig. 3a and b). This result could be due to the protocol used for cell preparation prior to immunization (see above). In contrast to NZ(pCYT : E7) and NZ(pCWA : E7), where all the E7 forms produced are found in the cell fraction, in NZ(pSEC : E7) E7 is released into the supernatant (discarded during sample preparation) (Fig. 2a). Thus, the amounts of E7 delivered in immunization with NZ(pSEC : E7) might be very small (only trace amounts of preE7 were detected in the cell fraction) if heterologous protein production and secretion stop once nisin is removed during and after sample preparation.

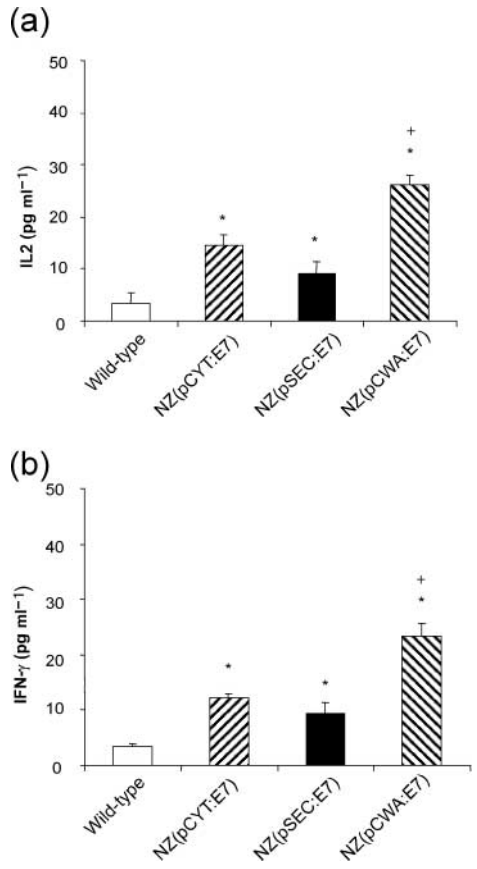

Fig. 3. Evaluation of immune response in mice immunized with the three recombinant *L. lactis* producing E7 in different cellular localizations. Levels of IL2 (a) and IFN-γ (b) cytokines were assayed by ELISA following immunization of mice with 1×10^9 c.f.u. of different *L. lactis* strains: wt, NZ(pCYT : E7), NZ(pSEC : E7) or NZ(pCWA : E7). Statistically significant differences ($P < 0{\cdot}05$) between the wt immunized group and NZ(pCYT : E7), NZ(pSEC : E7) or NZ(pCWA : E7) immunized groups are denoted by an asterisk (*). Significant differences between NZ(pCYT : E7), NZ(pSEC : E7) or NZ(pCWA : E7) immunized groups are denoted by a cross (+). These data are representative of three separate experiments showing similar results.

We therefore checked whether *L. lactis* continues to produce E7 antigen after a nisin pulse. Production and secretion of E7 by NZ(pSEC : E7) were analysed by Western blot and immunodetection. NZ(pSEC : E7) was induced with 10 ng nisin ml^{-1} for 1 h (nisin pulse). After the nisin pulse, cells were recovered and thoroughly washed three times with fresh culture medium (GM17) to eliminate all traces of nisin. After the last wash, the cell pellet was suspended in GM17 and growth was pursued for 6 h as described previously (Bermúdez-Humarán *et al.*, 2003c). Culture samples were taken at intervals of 2, 4 and 6 h and analysed by Western blot and immunodetection using anti-E7 antibodies (Fig. 4). Interestingly, the quantity of E7 protein in the washed sample 4 h after the nisin pulse was about twofold higher than in the sample prepared from non-washed culture just after the nisin pulse (Fig. 4). Similar results were recently observed with two other heterologous proteins, the staphylococcal nuclease and the ovine IFN-ω (Bermúdez-Humarán *et al.*, 2003c). This result could be due (i) to the toxic effect of nisin (nisin is a lactococcal bacteriocin, active on *L. lactis* strains) on NZ(pSEC : E7), since *L. lactis* NZ9000 strain does not possess a nisin resistance gene (de Ruyter *et al.*, 1996; Kuipers *et al.*, 1998), and/or (ii) to a linkage between nisin and its NisK receptor at the lactococcal surface that is resistant to the washing steps and leads to a persistent nisin induction. To check whether persistent E7 production was due to nisin

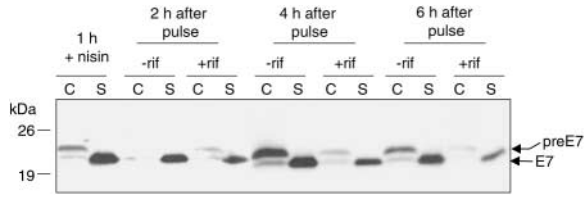

Fig. 4. E7 production after nisin pulse. After 1 h induction of strain NZ(pSEC : E7) with 10 ng nisin ml^{-1}, the cell pellet was recovered, washed and suspended in fresh GM17 with (+rif) or without rifampicin (−rif). Growth was pursued for 2, 4 and 6 h and E7 production was analysed by Western blot.

induction even after washing, the induced cultures were incubated with or without rifampicin, an antibiotic that blocks transcription. E7 production was analysed every 2 h for 6 h. The addition of rifampicin had a dramatic influence on E7 production. The amount of E7 detected 2 h after the nisin pulse in rifampicin-treated cultures was approximately twofold lower than in cultures without rifampicin. In the presence of rifampicin, E7 levels decreased to trace amounts a few hours after the nisin pulse (Fig. 4). These results suggest that, even after washing, some nisin remained linked to NisK receptors and continued to activate transcription from the P*nisA* promoter.

These results show that induced *L. lactis* continue to produce and secrete E7 even after sample preparation for intranasal administration. This is totally in accordance with previous results on the *in vivo* delivery of biologically active molecules by *L. lactis* strains administered intranasally to mice (Bermúdez-Humarán *et al.*, 2003b; Cortes-Perez *et al.*, 2003). As E7 is an extremely labile protein (Reinstein *et al.*, 2000; Bermúdez-Humarán *et al.*, 2002), the lack of significant immune response after immunization with NZ(pSEC : E7) could be due to rapid degradation of E7 once *in vivo* or to poor immunogenicity of E7 protein itself. It is well known that soluble E7 protein is poorly immunogenic and the use of adjuvants is crucial to evoke an immune response (Gérard *et al.*, 2001).

Perspectives on the use of *L. lactis* to treat HPV-related CxCa

We have previously shown that immunization of mice with an *L. lactis* strain displaying E7 antigen at its surface evoked a humoral response as corroborated by antibody production (Cortes-Perez *et al.*, 2003). Immune response mediated by antibodies is considered essential in infections. However, to date, the importance of the humoral response in CxCa remains unclear, even though a potential efficacy has already been suggested (Berumen & Villegas, 1997). In this work, we show that (i) *L. lactis*, a food-grade and non-pathogenic bacterium, can efficiently produce E7 antigen in different cellular locations and (ii) recombinant lactococci strains can induce an E7-specific CTL immune response in mice after intranasal administration. These encouraging results represent a step towards the development of a new, safe mucosal vector to treat HPV-related CxCa. Furthermore, as HPV infection is associated with other diseases (i.e. anogenital cancers, papilloma, warts and even some non-melanoma skin cancers) in addition to CxCa, recombinant lactococci engineered to express E7 could ultimately have an impact on other HPV-related diseases.

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