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Vaccine 22 (2004) 1079–1086
Vaccine
www.elsevier.com/locate/vaccine

Recombinant virus-like particles of a norovirus (genogroup II strain) administered intranasally and orally with mucosal adjuvants LT and LT(R192G) in BALB/c mice induce specific humoral and cellular Th1/Th2-like immune responses

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Received 23 January 2003; received in revised form 6 October 2003; accepted 15 October 2003

Abstract

We investigated the immune response induced by mucosal immunization of BALB/c mice with virus-like particles (VLPs) of a genogroup II norovirus, Dijon171/96 virus, produced in the baculovirus system. VLPs administered alone by the intranasal route induced a high serum antibody response as well as fecal IgA, which were enhanced when the heat-labile *Escherichia coli* toxin or its non toxic mutant LT(R192G) was coadministered. In these conditions, the oral route was also efficient. Cytokine production by cells from different lymphoid tissues was then assessed after in vitro restimulation. A Th1/Th2-like response was observed in cervical lymph node and Peyer's patch (PP) cell cultures from mice intranasally or orally immunized with either adjuvant indicating that, on the assumption that T cells are the primary cells producing the cytokines after in vitro restimulation, specific T lymphocytes are present in the intestine after intranasal immunization.

Keywords: Norovirus; VLPs; Immune response

1. Introduction

Norwalk-like viruses recently assigned as noroviruses [1] (*Caliciviridae* family) represent the most important cause of acute gastroenteritis outbreaks in industrialized countries [2,3] and are also now recognized as a frequent agent of gastroenteritis in the community in all age groups [4–6]. Noroviruses are divided into two genogroups: genogroup I (GI) and genogroup II (GII) which show genetic and antigenic differences. Expression of Norwalk virus (NV, GI) ORF2, encoding the major capsid protein, in insect cells infected with recombinant baculovirus [7] and in mammalian cells [8] yields self-assembled virus-like particles (VLPs) which are antigenically similar to the native particles. Because NLVs are difficult to produce, these VLPs represent an important source of antigen that can be used in place of the native virus to better understand immunity to NLVs [9]. Furthermore, rNV VLPs have been considered as a candidate vaccine for NV infections in humans on the basis of data obtained in mice [8,10–12] as well as in humans [13]. Because of the important genetic variability of the capsid protein of noroviruses, it may be important to include different VLPs in the vaccinal preparations to induce a more broadly immune response. In this study, we investigated the immune response to VLPs of Dijon171/96, a genogroup II Grimsby-like strain (Lordsdale genotype) expressed in the baculovirus system [14]. Both the humoral and cellular responses were investigated: the serum and fecal antibody responses were assessed in BALB/c mice inoculated intranasally with VLPs alone or in the presence of mucosal adjuvant, the heat-labile *Escherichia coli* toxin (LT) or its non toxic mutant LT(R192G), as well as in mice orally inoculated in the presence of either adjuvant. The cytokine response was assessed in cell culture supernatants of different lymphoid tissues from mice inoculated intranasally and orally in the presence of adjuvant as previously described for rotavirus VLPs [15]. The results showed that Dijon171/96 VLPs administered alone by the intranasal route induced a strong systemic humoral response

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doi:10.1016/j.vaccine.2003.10.004

as well as fecal antibodies both enhanced in the presence of LT or LT(R192G). In these conditions, the oral route was also efficient. In addition, oral and intranasal immunization of VLPs in the presence of LT or LT(R192G) induced a Th1/Th2-like cellular immune response by cells from lymphoid tissues close to or distant from the immunization site.

2. Materials and methods

2.1. VLP preparation and characterization

Dijon171/96 VLPs were prepared and purified as previously described [14] with Sf9 cells being replaced by High-Five cells. Briefly, a 1620 bp fragment corresponding to the entire *ORF2* gene of Dijon171/96 virus isolated from a stool specimen was cloned in the baculovirus transfer vector pVL1393 (Invitrogen). The ORF2 sequence predicted a 539 amino acids capsid which exhibited 98.7% nucleotide and 98.7% amino acid identity with Grimsby virus. High-Five infected cell supernatants were collected 5 days postinfection, extracted with Freon 113 before being concentrated by ultracentrifugation. The pellets were centrifugated through a 40% (w/w) sucrose cushion then through a preformed CsCl gradient (28–36% (w/w) in water: 1.2644–1.3661 g/cm^3) for 22 h at 35,000 rpm (LKB RPS56T rotor). Purified VLPs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to confirm their protein composition and examined by negative-staining electron microscopy. Protein concentration was determined using the Lowry method with BSA as a standard.

2.2. Immunization and sample collection

Adult female BALB/c mice (4–5 weeks old) were from Iffa-Credo (L'Arbresle, France) and from Harlan (France). Mice were immunized intranasally or orally with VLPs on days 0 and 14. Prior to intranasal inoculation, mice were anesthetized by intraperitoneal administration of a mixture of ketamine (80 mg/kg) and xylazine (16 mg/kg). The immunogen was given by gradual inoculation of the nostrils of the mice. Mice were immunized either intranasally or orally with 10 μg of VLPs mixed with 10 μg of LT (Sigma, St. Louis, MO) or LT(R192G) (kindly supplied by Dr. John D. Clements, Tulane University Medical Center, New-Orleans) per dose in a volume of 20 μl. In addition, one group of mice received 25 μg of VLPs without adjuvant by the intranasal route. Control mice were inoculated intranasally with phosphate-buffered saline (PBS) in the presence of LT or LT(R192G). Blood and fecal samples were collected from each mouse on day 35 (21 days after the second immunization) and stored at −40 °C prior to use. Mice were then sacrified, and the different lymphoid tissues, spleen, CLN, MLN or CLN and PP depending on the experiments were aseptically removed.

2.3. Measurement of Dijon171/96 virus-specific antibodies in serum and fecal samples by ELISA

Antibody titers in serum and fecal samples were determined by ELISA. Microtiter plates were coated overnight at 4 °C with 100 ng of VLPs in PBS. Wells were blocked with PBS containing 5% non fat dry milk. Fecal samples were made 10% (w/v) suspension in PBS, pH 7.4. Serial five-fold dilutions in PBS 5% non fat dry milk of serum (starting at 1/100) or two-fold dilutions of fecal extracts (starting at 1/40) were added to wells and incubated for 40 min at 37 °C. After three washes, the plates were incubated for 30 min at room temperature with a 1:5000 dilution of biotin-labeled goat anti-mouse α, γ, γ1, or γ2a heavy chain-specific antisera (Southern Biotechnology Associates Inc., Birmingham, AL). The plates were washed, and peroxidase-labeled avidin (Southern Biotechnology Associates) was added. The color reaction was developed at room temperature in the dark with the chromogenic substrate orthophenylenediamine with 0.03% H_2O_2, and A_{492} was determined. Endpoint titers were expressed as the reciprocal $\log_{10}$ of the last dilution that gave an optical density at least two-fold greater than the mean value obtained with samples from uninfected mice for the same dilution (cutoff value of 0.1). Negative serum and fecal samples (titer <100 and <40, respectively) were arbitrarily assigned titers of 50 and 20 (two-fold <100 and <40), respectively, for statistical calculations.

2.4. Preparation of spleen, CLN, PP and MLN cells and in vitro restimulation

Spleens, superficial and posterior CLN, MLN or CLN and PP depending on the experiments were removed and single-cell suspensions were prepared by mechanical dissociation. Cells (4×10^6 cells/ml) were resuspended in complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.3% glucose, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% heat-inactivated FCS). In a first set of experiments, spleen, CLN and MLN cells from mice intranasally inoculated with VLPs or PBS in the presence of LT (4×10^5 cells/well) were cultured in the presence of 5 μg of VLPs/ml or RPMI medium only in 96-well plates. The T-cell mitogen concanavalin A (5 μg/ml) was added to positive control wells. The cells were incubated at 37 °C with 5% CO_2. The culture supernatants were harvested at days 2, 3, 4, 5 and for spleen 2, 3, 4, 5, 6 after restimulation to determine the kinetics of production of the different cytokines. In a second set of experiments, CLN and PP cells from mice immunized intranasally or orally with VLPs in the presence of LT or LT(R192G) as well as CLN and PP cells from control mice inoculated intranasally with PBS in the presence of the different adjuvants were cultured as previously. The culture supernatants were harvested on days 3 and 5 after restimulation. All supernatants were frozen at −70 °C until cytokine analysis.

2.5. Cytokine ELISA

Cytokine levels in culture supernatants were determined by ELISA. IFN-γ, IL-2 and IL-4 levels were determined using monoclonal antibodies (Mabs) obtained from PharMingen (San Diego, CA). Mabs used for coating were clones R4-6A2 (2 μg/ml), JES6-1A12 (2 μg/ml) and 11B11 (2 μg/ml) and Mabs used for detection were clones XMG1.2 (0.5 μg/ml), JES6-5H4 (0.5 μg/ml) and BVD6-24G2 (0.5 μg/ml) for IFN-γ, IL-2 and IL-4, respectively. Standard curves were generated using recombinant murine IFN-γ, IL-2 and IL-4 (PharMingen). IL-5 levels were measured using a licensed kit (Endogen, Cambridge, MA) following the manufacturer's recommendations.

2.6. Statistics

Antibody responses were analyzed for comparison between the different groups with one-way analysis of variance, after verification of variance's homogeneity by using Hartley's table. For each antibody analyzed, in the case of a significant one-way analysis of variance test, post hoc analysis comparing results between the different experimental arms was conducted by using a Scheffé test. Comparison between the intranasal and the oral routes for a given adjuvant was performed using the Mann–Whitney nonparametric *U*-test. For all tests, a *P*-value of <0.05 was considered significant.

3. Results

3.1. Serum and fecal antibody responses in mice inoculated intranasally and orally with Dijon171/96 VLPs with and without LT or LT(R192G)

We examined the serum and fecal antibody responses in mice inoculated twice intranasally either with 25 μg of VLPs alone or with 10 μg of VLPs in the presence of 10 μg of LT or LT(R192G) as well as in control mice that received PBS with or without any adjuvant. No significant antibody response was observed in control mice. Mice immunized intranasally with 25 μg of VLPs alone developed high IgA and IgG serologic responses (Fig. 1A and B). We further characterized the systemic IgG response by assessing IgG1 and IgG2a subclass responses. Elevated IgG1 and IgG2a levels were observed (Fig. 1D). In fecal extracts, we could not detect any IgG responses whereas low levels of IgA were observed in all mice (Fig. 1C).

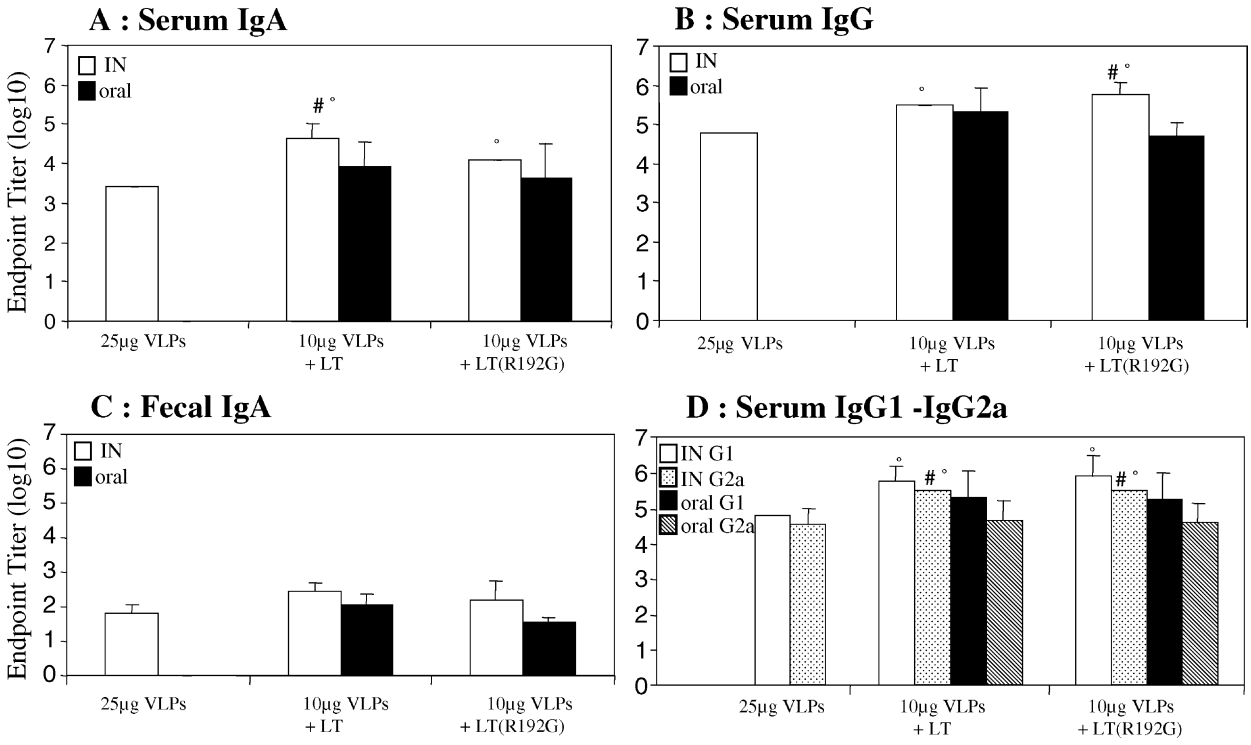

Fig. 1. Serum (A, B and D) and fecal (C) Dijon171/96 VLPs-specific antibody responses in mice inoculated twice intranasally with 25 μg of VLPs alone or twice intranasally or orally with 10 μg of VLPs and 10 μg of LT or LT(R192G). Serum anti-VLPs IgA (A), IgG (B), IgG subclass IgG1 and IgG2a (D) as well as fecal IgA titers (C) were determined 21 days after the second immunization. The results are plotted as the geometric mean titers for all groups, and error bars represent 1 standard error of the mean. VLPs-specific antibody responses were not detected in unimmunized mice. (O) Significant difference ($P < 0.05$) compared to the group given 25 μg of VLPs alone for the same isotype; (#) significant difference ($P < 0.05$) between the groups immunized intranasally or orally for the same isotype.

The coadministration of 10 μg of VLPs with 10 μg of LT or LT(R192G) by the intranasal route induced significantly higher titers of serum IgA and IgG than administration of 25 μg of VLPs alone ($P < 0.0001$, 0.004 for IgA; 0.0045 and 0.0008 for IgG responses in the presence of LT and LT(R192G), respectively; Fig. 1A and B). We further characterized the effects of LT or LT(R192G) on the IgG subclass response (Fig. 1D). Significantly higher titers of both IgG1 and IgG2a were observed when VLPs were administered in the presence of either adjuvant compared to the administration of 25 μg of VLPs alone ($P = 0.0008$, 0.0002 for IgG1 responses in the presence of LT and LT(R192G) and $P < 0.0001$ for IgG2a responses in the presence of either adjuvant). IgG1 and IgG2a titers were not different between the two adjuvants. In fecal extracts, IgA were higher with LT and LT(R192G) but not significantly different and IgG were not detectable (Fig. 1C). No significant difference in serum and fecal responses was observed between LT and LT(R192G).

Mice inoculated orally with 10 μg of VLPs in the presence of LT or LT(R192G) developed IgA and IgG serologic responses (Fig. 1A and B) with elevated IgG1 and IgG2a

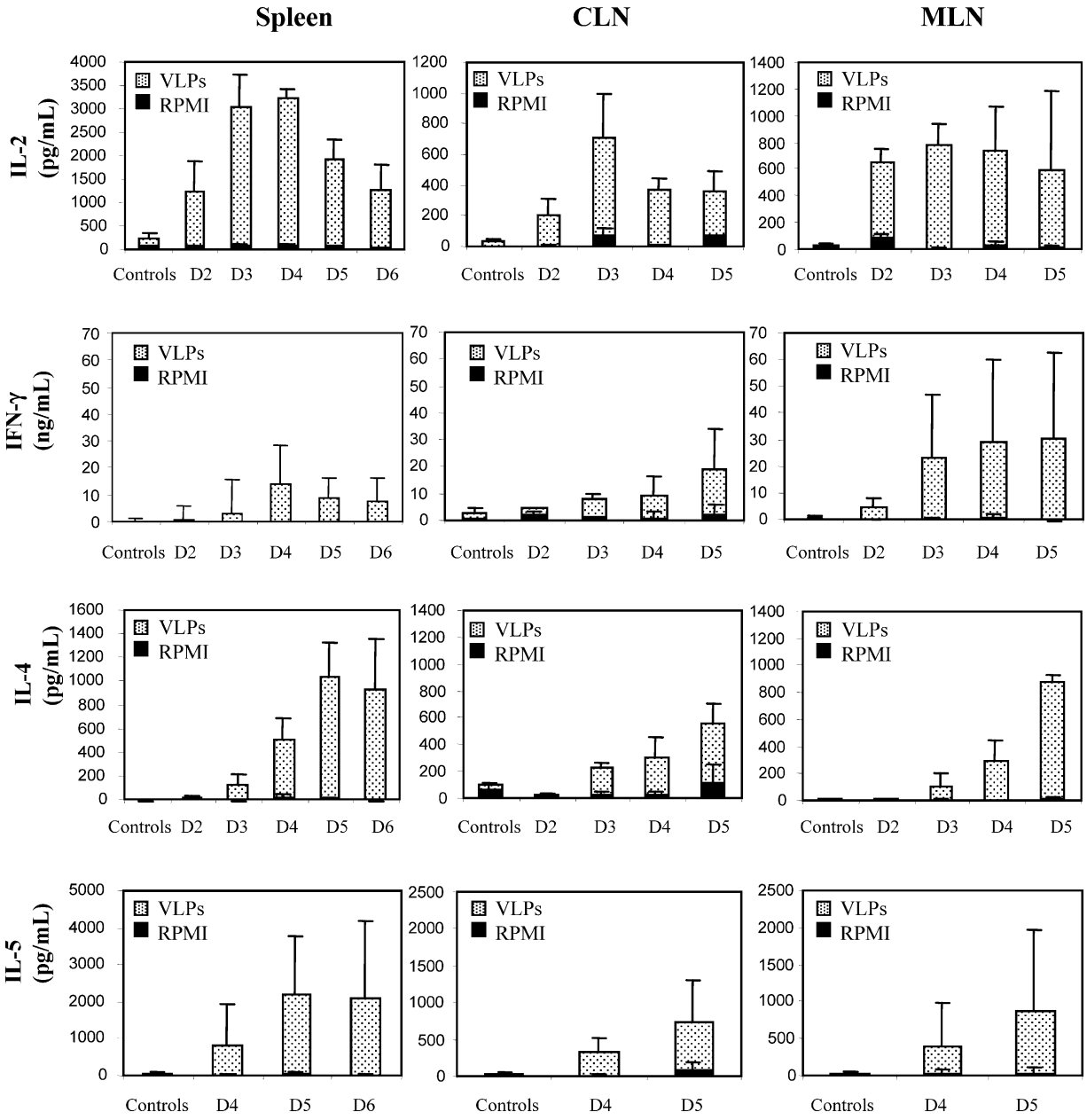

Fig. 2. Kinetics of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-5) cytokine production by spleen, CLN and MLN cells from mice immunized twice intranasally ($n = 3$) with 10 μg of VLPs and 10 μg of LT and from unimmunized mice ($n = 3$). Cells were removed 3 weeks after the second immunization and restimulated in vitro with VLPs or cultured in the presence of RPMI medium only. The capacity of cells to be stimulated in vitro and to produce cytokines was confirmed by production of cytokines in the presence of concanavalin A. Cytokine production was measured 2, 3, 4, 5 and for spleen 6 days after in vitro restimulation. Results are means for individual mice plus standard deviations.

levels (Fig. 1D). No significant difference was observed between LT and LT(R192G). In fecal extracts no IgG response was observed whereas low levels of IgA were observed (Fig. 1C). Finally mice immunized orally tended to develop lower levels of serum IgA, IgG, IgG1 and IgG2a as well as fecal IgA than mice immunized intranasally (Fig. 1A–D). However, the difference was significant only for serum IgA ($P = 0.048$) and IgG2a ($P = 0.09$) and for serum IgG ($P = 0.014$) and IgG2a ($P = 0.014$) with LT and LT(R192G), respectively.

3.2. Kinetics of cytokine production by spleen, cervical and mesenteric lymph node cells from mice intranasally immunized with Dijon171/96 VLPs in the presence of LT

We first examined the Dijon171/96 virus specific cytokine production by cells from mice intranasally inoculated twice with VLPs in the presence of LT. IL-2, IFN-γ, IL-4 and IL-5 were assessed at different times after in vitro restimulation with VLPs in cell culture supernatants from spleen and from cervical lymph nodes (CLN) where antigen-sensitized cells migrate after intranasal immunization. Cytokines were also investigated in mesenteric lymph node (MLN) cell culture supernatants. Residual production from in vivo priming was evaluated in wells incubated without antigen in the presence of RPMI medium only. Cells from control mice that received PBS with LT did not produce any significant cytokine titers after in vitro restimulation with VLPs and the results at different times were combined (Fig. 2). Spleen as well as CLN and MLN cells from immunized mice produced a mixed Th1/Th2-like cytokine profile including IL-2, IFN-γ, IL-4 and IL-5 (Fig. 2). IL-2 production peaked earlier (days 3–4) than IFN-γ and IL-4 and IL-5 production. Partial results obtained at D6 for CLN and MLN (data not shown) suggested

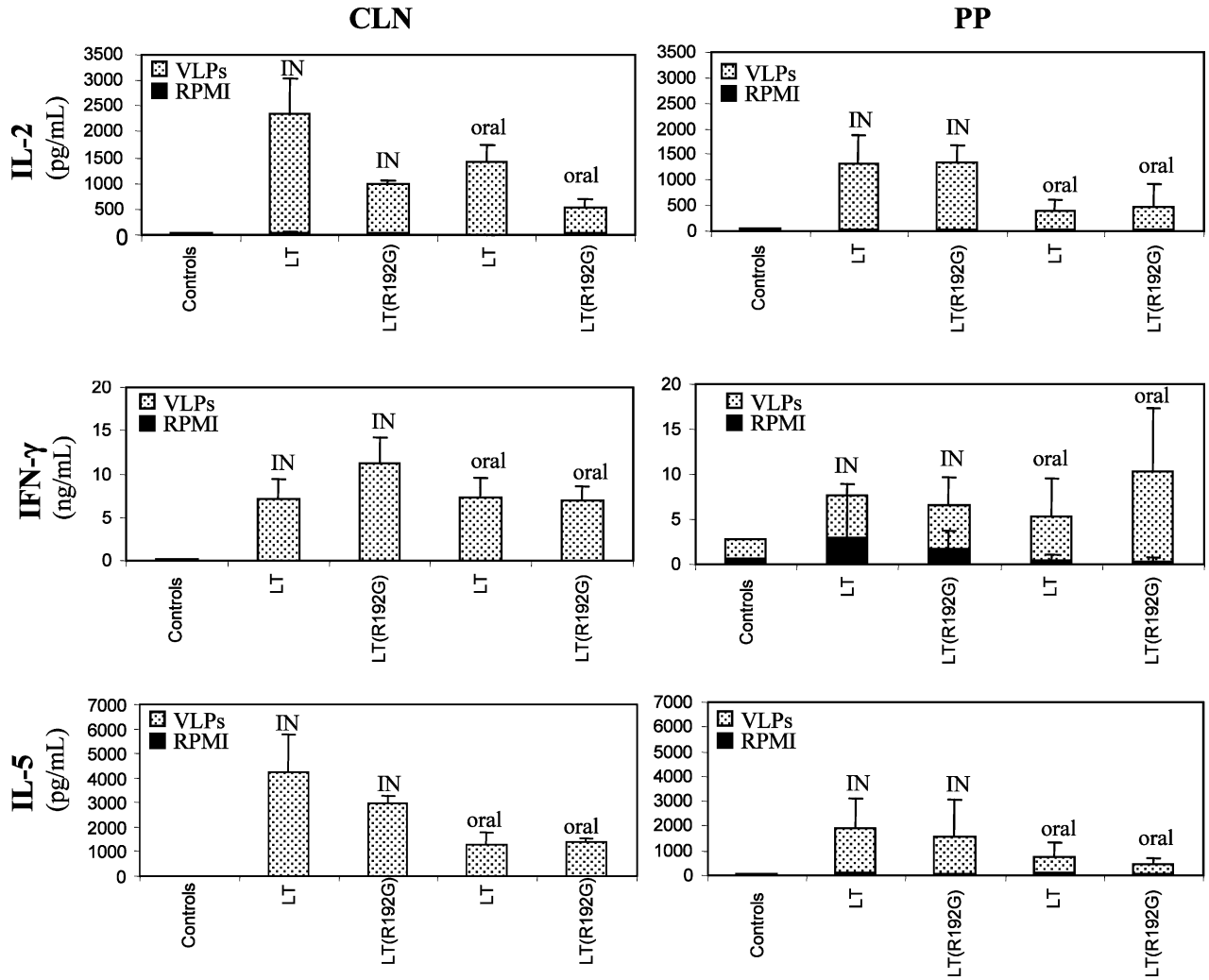

Fig. 3. IL-2, IFN-γ and IL-5 production by CLN and PP cells from mice immunized twice intranasally ($n = 3$) or orally ($n = 3$) with 10 μg of VLPs and 10 μg of LT or LT(R192G) and from unimmunized mice ($n = 3$). Cells were removed 3 weeks after the second immunization and restimulated in vitro with VLPs or cultured in the presence of RPMI medium only. The capacity of CLN and PP cells to be stimulated in vitro and to produce cytokines was confirmed by production of cytokines in the presence of concanavalin A. Results are means for individual mice plus standard deviations.

that IFN-γ, IL-4 and IL-5 levels had reached a maximum at D5.

3.3. Cytokine production by cervical lymph node and Peyer's patch cells from mice intranasally and orally immunized with VLPs in the presence of LT or LT(R192G)

We then examined IL-2, IFN-γ and IL-5 production, as being representative of Th1 and Th2, by cells from CLN and Peyer's patches (PP) (that represent the small intestine inductive sites from where cells migrate to the MLN then to the *lamina propria*) from mice twice inoculated intranasally or orally with VLPs and LT or LT(R192G). IL-2 was assessed on day 3, and IFN-γ and IL-5 on day 5 on the basis of the first experiments performed with spleen and CLN and of a preliminary experiment with PP which had shown that the kinetics was comparable.

CLN and PP cells from control mice inoculated intranasally with PBS and one of the adjuvants did not produce IL-2 nor IL-5 after in vitro restimulation whereas a low spontaneous production of IFN-γ was observed in PP culture supernatants from some mice in both groups. The results for the two groups were combined (Fig. 3).

CLN as well as PP cells from mice inoculated intranasally with VLPs and LT or LT(R192G) produced IL-2, IFN-γ and IL-5 after in vitro restimulation with VLPs, thus confirming the previous results obtained with LT (Fig. 3).

PP cells as well as CLN cells from mice inoculated orally with VLPs and LT or LT(R192G) produced also a mixed Th1/Th2-like cytokine profile including IL-2, IFN-γ and IL-5 after in vitro restimulation with VLPs. No evident difference was observed between the two adjuvants. Equal amounts of each cytokine and of IL-5 were produced in PP and CLN cell cultures, respectively, and wheras IL-2 tended to be lower in CLN cell culture after intranasal and oral immunization in the presence of LT(R192G), higher or equal amounts of IFN-γ were observed, which did not allow to conclude to a higher Th1 response with LT.

Finally, compared to mice intranasally inoculated, IL-2 and IL-5 production by cells from mice orally inoculated was lower whereas IFN-γ titers were more similar.

4. Discussion

Recent studies have shown that rNV VLPs (genogroup I) inoculated orally to BALB/c and to CD1 mice as well as intranasally to BALB/c mice induced significant fecal and serologic humoral responses which may be strongly enhanced in the presence of the mucosal adjuvants cholera toxin (CT) and the mutant *E. coli* heat-labile toxin LT(R192G) [10,12]. In the present study, we investigated both the specific humoral and cytokine responses induced by intranasal and oral immunization of BALB/c mice with recombinant VLPs of Dijon171/96 virus, a Grimsby-like strain belonging to genogroup II. VLPs administered without any adjuvant by the intranasal route at the dose of 25 μg induced a strong systemic IgG and IgA response as well as fecal IgA and higher titers of antibodies were observed when a lower dose (10 μg) of VLPs was administered in the presence of the mucosal adjuvant LT or LT(R192G). These results are in accordance with results obtained with GI VLPs administered alone and in the presence of LT(R192G) by the intranasal route [10]. No significant difference in serologic IgG, IgA and fecal responses was observed between the two adjuvants, notably, the non toxic mutant of LT, LT(R192G), did not induce lower titers compared to LT.

Mice orally immunized with 10 μg of VLPs in the presence of LT or LT(R192G) developed high IgG and IgA serologic responses as well as fecal IgA. No significant difference was observed between the two adjuvants. Compared to the intranasal route, lower levels of serum IgG and IgA were observed as well as fecal IgA, however, the difference was not significant in all cases. These results show that the oral route is efficient to induce a significant humoral response even when low doses of VLPs are administered. They show some discrepancies with results reported by Guerrero et al. and by Harrington et al. which showed that low doses (10 μg) of rNV VLPs produced in the baculovirus system were not immunogenic [10] and that purified rNV VLPs expressed in a Venezuelan equine encephalitis replicon system (VRP) failed to induce serum and intestinal antibody responses even at high doses [8] whereas VRP expressing the NV capsid protein administered subcutaneously induced a strong serum and fecal antibody response. Periwal et al. [16] have shown recently that rNV VLP administered by the oral route after pretreatment with sodium bicarbonate resulted in a much better immune response, suggesting that low pH may lead to some VLP degradation. However, the IgG response obtained with rNV VLP after pretreatment was still lower than the response induced by the Dijon171/96 VLPs whereas comparable results were observed for the intranasal route. A higher stability of Dijon171/96 VLPs to low pH and/or to proteolytic enzymatic digestion may result in a better conservation of immunogenicity in the digestive tract.

The specific cytokine response was assessed both in CLN and PP after intranasal and oral immunization in the presence of adjuvant. CLN represent the regional lymph nodes where lymphocytes sensitized in the NALT migrate after intranasal administration and PP the inductive site after oral administration, from which sensitized lymphocytes migrate to the MLN then to the *lamina propria*. It is of particular importance to assess the immune response in the intestine after immunization by the intranasal route since noroviruses are enteric pathogens and the immune responses are expected to be stronger at the site of induction than at distant sites. A specific Th1/Th2-like cytokine response including IL-2, IFN-γ and IL-5, in accordance with the fact that both IgG1 and IgG2a, were produced, was observed after in vitro restimulation of CLN as well as PP cells from mice intranasally immunized with VLPs and either adjuvant. This

mixed Th1/Th2 response was also observed in spleen and mesenteric lymph nodes of mice immunized in the presence of LT and in these conditions, IL-4 was also produced. These results are in accordance with results obtained previously with 2/6 rotavirus VLPs [15]. Indeed, we showed that 2/6 rotavirus VLPs administered intranasally in BALB/c mice in the presence of LT induced a mixed Th1/Th2 response in spleen, CLN, MLN and PP cell cultures from mice immunized in the presence of LT. Since after in vitro restimulation, cytokines are most likely produced by T cells, we concluded that rotavirus-specific lymphocytes were present in the intestine after intranasal immunization. In a similar manner, the results obtained here suggest that lymphocytes specific for Dijon171/96 VLPs are present in the intestine after intranasal inoculation of these VLPs. These results are in accordance with recent results reporting the presence of memory T cells in intestinal lymphoid tissue after intranasal immunization with the protein SAG1 of *Toxoplasma gondii* with CT [17], *Cryptosporidium parvum* DNA [18] or hen egg lysozyme in the presence of adjuvant [19].

A balanced Th1/Th2 response has generally been reported after oral or intranasal immunization in the presence of LT [20–22]. Furthermore, the non toxic mutant LT(R192G) has also been shown to induce Th1 and Th2 cytokine responses notably by spleen cells from BALB/c mice intranasally immunized with HIV-gp160 [23] and by spleen and PP cells from BALB/c mice orally immunized with inactivated *influenza* vaccine [24]. In this case, no difference in the response was observed between LT and its non toxic mutant. Here, we did not observe any evident difference between LT and its non toxic mutant. Indeed, equal amounts of each cytokine were produced in PP cell cultures and, although some differences were observed in CLN cell cultures for Th1 cytokines, they did not allow to conclude to a higher Th1 response with any of the adjuvants.

Finally, in accordance with the fact that both IgG1 and IgG2a were produced, the same profile was also found when the oral route was used, however, IL-2 and IL-5 production tended to be lower. Nevertheless, compared to results obtained with 2/6 rotavirus VLPs administered by both routes at the same dose [15], a better response was observed after oral inoculation of Dijon171/96 VLPs compared to the intranasal route suggesting a higher stability of these VLPs in the digestive tract.

In conclusion, we have shown that Dijon171/96 VLPs (genogroup II) as rNV VLPs (genogroup I) were immunogenic when administered to BALB/c mice by the intranasal and the oral route in the presence of mucosal adjuvant. Both routes of immunization, the intranasal route being nevertheless more efficient, resulted in a humoral response including serum and fecal antibodies as well as in a Th1/Th2-like cytokine response at sites close to or distant from the immunization site, notably in the intestine after intranasal immunization. On the assumption that T cells are the primary cells producing the cytokines after in vitro restimulation, specific T lymphocytes are present in the intestine after intranasal immunization with such VLPs. Recent results have highlighted the importance of CD4 T cells, the only lymphocytes needed to protect mice against rotavirus shedding after intranasal immunization with a chimeric VP6 protein and LT(R192G) [25]. The Th1/Th2-like cellular response observed in this study may be also important for protection after immunization with calicivirus VLPs. An effective vaccine against noroviruses would be useful for at-risk population such as health care providers, military personnel, and food handlers. However, because of the lack of information about immunity to noroviruses, the absence of an animal model of infection and the great variability of these viruses, the best approach to develop a vaccine against noroviruses has not yet been determined. VLP are currently being examined as candidate vaccines for a number of other viral pathogens including notably rotavirus [26] and have been shown to induce both T and B cell effectors as well as protection when administered in the presence of mucosal adjuvant. In addition, in the case of rotavirus, they have been shown to induce cross-protective immunity against heterotypic strains [27,28]. The study we conducted here provides information on the immune effectors induced by oral and intranasal administration of such a nonreplicating candidate norovirus vaccine in the presence of mucosal adjuvant.

Acknowledgements

We thank Dr. John D. Clements, Tulane University Medical Center (New-Orleans) for kindly providing LT(R192G) for this study.

This work was supported by grants from the Conseil Regional de Bourgogne, the Ministère de la Recherche and ECOS-Nord (no. CF99S01).

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