# Immunization with Cry1Ac from *Bacillus Thuringiensis* Increases Intestinal IgG Response and Induces the Expression of FcRn in the Intestinal Epithelium of Adult Mice

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#### Abstract

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We have shown that Cry1Ac protoxin from Bacillus thuringiensis is a potent mucosal and systemic immunogen with adjuvant properties. Interestingly, we have observed that Cry1Ac preferentially induces high specific IgG responses in intestinal fluid when it is intraperitoneally administered to mice; therefore, in the present study, we used this protocol, as a model to address the influence of systemic immunization on the induction of the intestinal IgG response. The data shown indicate that upon intraperitoneal immunization with Cry1Ac, significant intestinal specific IgG cell responses were produced in the lamina propria, accompanied by an increased frequency of intestinal IgG+ lymphocytes and epithelial cells containing IgG. Considering that FcRn is the receptor responsible for the transport of IgG in neonatal intestinal epithelia, but it is developmentally downregulated in the rodent intestine, we analysed whether upon intestinal IgG induction, FcRn mRNA expression was induced in intestinal epithelial cells, of adult mice. Whereas in intestinal epithelia of unimmunized adult mice FcRn mRNA was not detected, in Cry1Ac immunized mice it was expressed, although the level was lower in comparison with that found in neonatal epithelia. Then using flow cytometry and immunofluorescence we confirmed that the expression of the protein FcRn was induced in the intestines of adult immunized mice especially in the large intestine. Finally, we found that Cry1Ac also increased FcRn expression in isolated intestinal epithelial cells stimulated in vitro. The outcomes suggest that the expression of FcRn in intestinal epithelium might be reactivated upon immunization, and possibly facilitate IgG transport.

### Introduction

IgA is the predominant immunoglobulin presented on mucosal surfaces, but, in addition to secretory IgA, significant quantities of IgG can also be induced and secreted into the intestinal lumen of adult humans and rodents [1]. Although the role of IgA in mucosal immunoprotection is well established, other isotype immunoglobulins such as IgG and IgM may also participate in mucosal protection [2]. Mucosally associated IgG has also been recently suggested to contribute to host defense based on evidence indicating that mucosal IgG Abs may act by neutralizing toxins, adhesins or viruses and by inhibiting the penetration of viruses across the epithelial layer [3]. Indeed, mucosal IgG induced by systemic immunization (e.g. with polysaccharide antigens from *Haemophilus influenzae*) or passive systemic administration of specific IgG antibodies (e.g. against simian immunode-ficiency virus) provides excellent protection against pathogens encountered on mucosal routes (respiratory or vaginal mucosa, respectively [1]. Likewise, within the lumen, IgG Abs can exert a protective function against HIV [4]. Moreover, IgG and IgM mucosal immunity to influenza in an IgA knockout mouse model has also been reported [5].

In contrast to all that is known about the active process of unidirectional IgA transport into the lumen across epithelial cells mediated by the polymeric Ig receptor (pIgR) [6] and its relationship to mucosal host defense against several pathogens, the contribution of intestinal luminal IgG to host defense, as well as the mechanisms by which IgG reaches luminal secretions and its function in these locations are not entirely known. The neonatal Fc receptor for IgG (FcRn) is the receptor responsible for mediating IgG transport across epithelial barriers. In addition, FcRn binds IgG and albumin in a pH-dependent manner and protects these from catabolism by diverting them from a degradative fate in lysosomes, through its expression on endothelia and potentially other cell types [7, 8]. Besides, FcRn can also enhance IgG-mediated antigen uptake across mucosal epithelia [9]. In mice and rats, FcRn is expressed at high levels in the intestinal epithelial cells of suckling pups where it is responsible for the transport of IgG present in maternal milk across epithelial cells and into the digestive circulation of the newborn animals; but, it is developmentally downregulated in the rodent intestine- as is highly expressed at birth, although a dramatic decrease in its expression within the epithelium (approximately 1000fold) occurs at the time of weaning (approximately 14 days of age), at the time of epithelial closure and, simultaneously, with the cessation of IgG transport [7, 10]. Although this phenomenon accounts for the ascription of 'neonatal' for this particular Fc receptor, it has recently been appreciated that FcRn continues to be expressed during adult life in humans, pigs, cows, monkeys and even rodents [11-14]. Indeed, human FcRn continues to be expressed in numerous adult human cell types including intestinal, kidney and bronchial epithelial cells [12, 15, 16]. The fact that FcRn is not expressed in the intestinal epithelia of adult mice suggests that intestinal IgG transport in adult rodents is not mediated by this receptor; however, it has not been explored whether FcRn is expressed in intestinal epithelia upon intestinal IgG induction.

In the present study, we elected the recombinant Cry1Ac protoxin from Bacillus thuringiensis ssp. kurstaki HD-73 as an immunogen to investigate intestinal IgG responses in adult mice. Insecticidal proteinaceous crystals named Cry proteins are produced as protoxins by Bacillus thuringiensis (Bt) during sporulation. Upon ingestion, crystalline protoxins are solubilized and proteolytically activated by midgut proteases of susceptible insects. The activated toxin, which is not toxic to vertebrates, binds to specific receptors on the brush-border membrane surface of the insects' midgut epithelium, inducing formation of pores and eventually leading to insect mortality [17]. In particular, Cry1Ac is a pore-forming protein that is specifically toxic to lepidopteran insect larvae by binding to the cell-surface receptor aminopeptidase N in Manduca sexta midgut via the sugar N-acetyl-D-galactosamine (GalNAc) [18, 19].

Although most of the studies on Cry proteins have been performed with regard to their toxicity in insects, we have described that recombinant Cry1Ac protoxin from *Bacillus thuringiensis* is a potent mucosal and systemic immunogen with adjuvant properties [20, 21]. Interestingly, we have observed that Cry1Ac preferentially induces high IgG responses in the fluids of both the large and small intestines when it is intraperitoneally administered to mice [22].Therefore, in the present study, we used this systemic immunization protocol, as a model to further characterize the induction of intestinal IgG response, as well as to address its influence on the expression of FcRn in intestinal epithelia.

To this aim, we first tested if the specific IgG response induced in intestinal secretions by i.p. Cry1Ac immunization was locally produced by lamina propria lymphocytes isolated from the small and large intestine by examining the anti-Cry1Ac antibody cell responses induced by ELISPOT. We also analysed the frequency of intestinal IgG+ B lymphocytes by flow cytometry and the expression of IgG in intestinal sections using immunohistochemistry. Interestingly, the immunohistochemical analysis revealed that some epithelial cells displayed IgG staining, suggesting that IgG might be being transported through the epithelia. Using RT-PCR we tested whether immunization induced the expression of FcRn mRNA in the intestinal epithelia of adult mice. Finally, the detection of the protein FcRn was determined by flow cytometry and immunofluorescence. The data shown indicate that intraperitoneal immunization with Cry1Ac induces the local production of specific IgG, increases the numbers of epithelial cells containing IgG and induces FcRn expression in the intestinal epithelia of adult mice.

#### Methods

*Recombinant Cry1Ac protoxin.* Recombinant Cry1Ac was purified from IPTG-induced *E. coli* JM103(pOS9300) cultures [23] as described previously [20, 21]. Endotoxin levels in the purified Cry1Ac protoxin preparations were tested using the E-toxate, Part 1 kit (Sigma Chem Co., St Louis, MO, USA) and were below 0.1 EU/ml, but they were further treated with an excess of a polymixyn B resin (BioRad, Hercules, CA, USA) to remove any possible remnants of endotoxin.

Animals. Male Balb/c mice (8–11 weeks of age) were used throughout this study. Animals were maintained in filter-topped cages, provided with sterile food and water *ad libitum*, and cared for according to the guidelines of the Federal Regulations for Animal Experimentation and Care (NOM-062-ZOO-1999; Ministry of Agriculture, Mexico).

*Immunizations.* Mice were i.p. immunized with 50  $\mu$ g of purified Cry1Ac protoxin in 0.1 ml phosphate buffered saline (PBS) on days 0, 7, and 14. Mice were sacrificed 7 days after the last immunization.

*Sample collection.* Blood was withdrawn by cardiac puncture of anesthetized mice. Intestinal lavages were harvested as previously described [24]. Briefly, the small and large intestines were flushed out, with 5 and 3 ml of

cold RPMI medium respectively, and were stored at -70 °C until use.

Determination of anti-Cry1Ac antibody levels in intestinal fluids. Intestinal anti-Cry1Ac antibody responses were measured by indirect ELISA as described [25]. Briefly, plates were coated with 100  $\mu$ l of Cry1Ac (10  $\mu$ g/ml). Non-specific binding sites were blocked with PBS containing 6% non-fat milk. Intestinal fluid samples, applied in volumes of 100  $\mu$ l per well were serially diluted with 0.05% PBS-Tween 20. Bounded antibodies were detected using either goat anti-mouse IgG-HRP (horseradish peroxidase) (1:2000; Pierce, Rockford, IL, USA), goat anti-mouse IgM-HRP (1:2000; Pierce), goat anti-mouse IgA-HRP (1:1000; Zymed Laboratories Inc., CA, USA), or anti-mouse IgE-HRP (AbD Serotec, Raleigh, NC, USA) conjugates. Colour reactions were developed using 0.4 mg/ml orthophenylendiamine in 0.05 M citrate-phosphate buffer, pH 5.2 supplemented with 0.01% H<sub>2</sub>O<sub>2</sub>. The reactions were stopped by adding 25 µl of 2 м H<sub>2</sub>SO<sub>4</sub> 15 min later. Optical densities were measured at 492 nm using a microplate reader (Ascent, Thermo Labsystems, Helsinsky, Finland). Titers were defined as the reciprocal of the highest endpoint sample dilution with an OD value 0.1 higher than the background. Total antibody levels were estimated using a doubleantibody sandwich ELISA (Zymed Laboratories). Concentration of total IgG in intestinal samples diluted 1:2 was quantified by interpolation of the OD values to a standard reference calibration curve of IgG (Sigma Chem Co.).

Intestinal lymphocyte and epithelial isolation. Intraepithelial and lamina propria lymphocytes were isolated and prepared according to a modification of a previously published method [26]. For intestinal epithelial cell isolation, discontinuous 20%, 40% and 70% Percoll gradients were used. The intestinal cells recovered after EDTA treatment was centrifuged at 453 g for 30 min at room temperature. Epithelial cells were collected from the interface following centrifugation over a 20–40% discontinuous Percoll gradient. Cells suspensions were adjusted to  $1 \times 10^6$  cells/ml in RPMI medium containing antibiotics and 5% fetal bovine serum (FBS).

*ELISPOT assay.* Individual Cry1Ac specific Ab-secreting cells were enumerated by a modified ELISPOT assay [27, 28]. Briefly, nitrocellulose discs were placed in the bottom of 24-well plates (Millipore, Bedford, MA, USA) and coated overnight at 4 °C with Cry1Ac at a concentration of 10  $\mu$ g/ml (500  $\mu$ l per well) in PBS. As negative control for antibody specificity, in ELISPOT assay nitrocellulose membranes were precoated with bovine serum albumin (BSA) instead of Cry1Ac. All wells were then blocked with 6% non-fat milk in PBS. At this point, 500  $\mu$ l of the cell suspension (1 × 10<sup>6</sup> cells/ml) in RPMI medium containing 5% fetal calf serum was added to each well and incubated for 3 h at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. The plates were thoroughly washed with PBS–Tween and then incubated for 2 h at room temperature with 500  $\mu$ l of the above indicated anti-mouse: IgA, IgM, IgG or IgE conjugated with horseradish peroxidase secondary antibodies. Unbound conjugates were removed by another series of washings and, finally, 500  $\mu$ l substrate [a buffered solution containing 0.01% H<sub>2</sub>O<sub>2</sub> and 0.5 mg/ml 4-cloro-naphtol (Sigma Chem Co.)] was added. The spots were counted with the aid of a top-illuminated dissecting microscope. Results are expressed as the averaged value of duplicate wells normalized to the estimated number of spots per 10<sup>6</sup> cells.

FACS analysis. For the characterization of B cells, intraepithelial lymphocytes and lamina propria lymphocytes prepared as described [26] were suspended in 0.5% BSA (Sigma Chem Co.) dissolved in PBS at a concentration of 10<sup>6</sup> cells/ml, followed by incubation at 4 °C in the dark with 20  $\mu$ l of properly diluted mAb for 30 min. Cells were washed with 0.5% BSA-PBS, suspended in 400 µl of 1% p-formaldehyde in PBS, and stored in the dark at 4 °C until analysed. MoAbs used for flow cytometry analysis were as follows: MoAbs antimouse, IgE-fluorescein isothiocyanate (FITC) (Biosource, Nivelles, Belgium), IgG-FITC, IgA-FITC, IgM-PE, B220-PE, CD3e-PerCP or FITC (BD-Pharmigen, St Jose, CA, USA), and chicken anti-mouse IgG Alexa fluor 594conjugated (Molecular Probes, Eugene, OR, USA). Appropriate Isotype controls were used (BD-Pharmigen). For detection of IgG or FcRn positive epithelial cells were utilized: FITC-conjugated Mab anti-E-cadherin (BD-Pharmigen), Alexa Fluor <sup>594</sup> labelled (Molecular Probes) or biotinilated anti-mouse IgG antibody, streptavidin PerCP (BD-Pharmingen), goat anti-FcRn (Santa Cruz Biotechnology, Inc) and anti-goat IgG Cy5 (Zymed Laboratories). Antibodies to cell surface markers were added to aliquots of the epithelial cells and, after incubation, the cells were washed and incubated with Cytofixcytoperm solution (Becton Dickinson, St Jose, CA, USA). Anti-IgG antibody was then added to perform intracellular staining, followed by washes with perm wash solution. To perform flow cytometric analyses, the relative fluorescence intensities were measured with a FACScalibur flow cytometer and analysed with BD Cell Quest Pro software v.5.1.1. Data were collected for 50,000 events. Lymphocyte and epithelial cell analysis was performed by two, three or four-colour immunofluorescence. The percentage of cells labelled with each mAb was calculated in comparison with cells stained with the appropriate isotype control antibodies. B and T cells were analysed within a lymphocyte gate defined by forward and side light scatter. For the analysis of epithelial cells, an enlarged gate that excluded the lymphocyte gate was used. The data represent the mean ± SD from three to five experiments using cells of a given tissue from two or three individually processed mice. Results are expressed as percentage of positive cells.

Immunohistochemistry and immunofluorescence. Tissue slices 5-6 mm thick were fixed in a freshly prepared ZSF solution (0.1 M Tris base buffer with Ca acetate 0.05% [pH 7-7.4] containing 0.5% Zn acetate and 0.5% Zn chloride), as described [29]. Tissue slices were dehydrated in graded alcohol and included in paraffin. A direct immunoperoxidase technique was used for IgG detection on mounted five micrometer thick sections. Briefly, endogenous peroxidase activity was quenched with 3% methanol/hydrogen peroxide and non-specific binding was blocked with 3% BSA/0.05% Triton X 100 in 0.01 M PBS, pH 7.0, for 30 min. The sections were incubated for 1 h with HRP labelled goat anti-mouse IgG (Pierce) diluted 1:500. Peroxidase activity was revealed with H<sub>2</sub>O<sub>2</sub>-diaminobenzidine (DAB; Sigma). The samples were counterstained with Harris' Haematoxylin, and then dehydrated and covered with Entellan (Merck, Darmstadt, Germany). For immunofluorescence staining, the blocked sections were first incubated with goat anti-FcRn antibody (1:500 in PBS; Santa Cruz Biotechnology), followed by incubation with the secondary antibody rabbit anti-goat IgG-Cy5 (1:500 in PBS; Invitrogen, Carlsbad, CA, USA) and finally with anti-mouse IgG-FITC (1:500 in PBS) (Pierce). Control experiments for staining specificity included appropriate isotype-matched non-specific antibodies or replacement of primary antibodies with non-immune serum and omission of primary antibody from incubation protocols. No specific staining was seen in either case. Immunofluorescence images were acquired using confocal laser scanning microscope (TCS SP2; Leica Microsystems AG, Wetzlar, Germany).

Messenger RNA analysis. We performed RT-PCR analysis to determine the mRNA expression level of mouse FcRn. After isolation, epithelial cells were collected and total RNA was extracted with TRIzol (Invitrogen) and precipitated with isopropyl alcohol and ethanol. RNA concentration was determined by absorbance at 260 nm, and single-stranded cDNA was synthesized from 1  $\mu$ g of total RNA by reverse-transcription reaction with 500 units of M-MVL RT (Promega, Madison, WI, USA). To generate a DNA probe for the alpha chain of FcRn, PCR primers were synthesized by Invitrogen Life Technologies, based on the previously reported mouse sequence for mRNA (GenBank NM\_010189). FcRn relative expression was evaluated in PCR amplification using the following sense and antisense primers: 5'-CAGCCTCTCACTGTGGACCTAGA-3' and 5'-AAAGCCATGGGGGCTGGCAGACC-3'. The amplification was accomplished by incubating 1  $\mu$ l of the resulting cDNA in a 30 µl reaction volume (50 mmol/l KCl, 150 mmol/l MgCl2, 10 mmol/l Tris-HCl, pH 9.0) containing 100 pmol of specific sense and antisense primers and 0.25 µl of Taq polymerase (Promega). PCR procedure comprises one heating cycle at 95 °C for 5 min followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, extension at 72 °C for 30 s, and one cycle of 72 °C for 1 min, for a product of 150 bp in length. The samples were analysed by duplicate on agarose gels and were quantified by densitometry using a Fuji-Film fluorescent Image Analyser FLA-5000 (Tokyo, Japan). The abundance of mRNA encoding FcRn was normalized by the abundance of mRNA encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as internal standard.

In vitro effect of Cry1Ac on FcRn expression in epithelial intestinal cells. Epithelial cells from the large and small intestines were isolated as indicated above from three control mice. The number of isolated epithelial cells from each intestine was adjusted to  $2 \times 10^6$  cells/ml with culture medium. Culture medium consisted consisted on RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM glutamine, 25 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt, 100 µg/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) (Gibco). Cells were cultured in 1 ml of culture medium with or without Cry1Ac (20  $\mu$ g/ml) in duplicates at 37 °C in 5% CO<sub>2</sub> in 24 well plates, for 3 and 24 h. The cells were harvest and stained with anti-FcRn and E-cadherin antibodies and analysed by flow cytometry as described above. The epithelial cultures were maintained renewing the medium every 48 h, and the experiment was repeated after 7 days, in cells cultivated in medium alone or stimulated with Cry1Ac for the last 24 h.

#### Results

### Cry1Ac induces increased levels of total and specific IgG responses in intestinal lavages

To address the influence of systemic immunization on the induction of the intestinal IgG response, we first examined the levels of specific and total antibodies in intestinal secretions of mice immunized with Cry1Ac by the i.p. route in comparison to those presented in control mice. As expected, we found that significant IgG anti-Cry1Ac antibody titers were induced almost exclusively in the lavages from the small and large intestine of immunized mice. Regarding total antibody levels, we observed that Cry1Ac immunized mice presented higher levels of IgG in intestinal lavages than unimmunized mice (P < 0.05). A quantitative ELISA confirmed that the concentration of IgG was significantly higher in the intestinal lavages from Cry1Ac immunized mice than in unimmunized mice (Fig. 1).

### Immunization with Cry1Ac induces specific antibody cell responses in the lamina propria

To determine if the anti-Cry1Ac IgG antibodies induced in the intestinal lavages of immunized mice were locally produced, lamina propria lymphocytes were isolated



Figure 1 Anti Cry1Ac titers and total antibody levels in the fluids from the small and large intestines of control and immunized mice. Cry1Ac: immunized mice that received three intraperitoneal doses of 50  $\mu$ g of Cry1Ac; control: untreated mice. Above: anti Cry1Ac antibody titers. Below panel: total antibody levels. SI: small intestine, LI: large intestine. Mucosal specific antibody titers and antibody levels were determined by ELISA as described in the Methods. Intestinal fluids were collected with 5 and 3 ml volumes, for the large and small intestines respectively. Anti Cry1Ac titers are expressed as the last dilution giving an OD 492 value of 0.2 (twofold higher than the mean background value). Total antibody levels are shown for a mucosal sample dilution of 1:2. Bottom left: Concentration of IgG values were determined in samples diluted 1:2. Results are expressed as the arithmetic mean  $\pm$  SD. n = 5 animals per experimental group. P < 0.001.

from the small and large intestine and the anti-Cry1Ac antibody cell responses were evaluated using ELISPOT. Specific antibody cell responses were induced in the lamina propria of both intestines, but higher numbers of IgA and IgM anti-Cry1Ac cell responses were recorded in the small intestine compared with the large intestine, where similar numbers of IgA, IgG and IgM cell responses were elicited (Fig. 2). Although, we found that the number of anti-Cry1Ac IgG-producing cells induced in the lamina propria of both intestines were not higher than those producing other isotypes, we observed that the spots of IgG-producing cells were bigger and more intense than the spots of the other isotypes, suggesting that its antibody production was higher (Fig. 2). In control unimmunized mice were not detected significant anti-Cry1Ac producing cells of any isotype in lamina propria (data not shown).

## Cry1Ac increases the frequency of IgG+ lymphocytes in the intestinal lamina propria

We determined whether the frequency of isolated intraepithelial and lamina propria intestinal  $Ig_+$  B lymphocytes was modified by immunization with Cry1Ac by flow cytometry analysis. Interestingly, we found that the proportion of intestinal lamina propria B lymphocytes expressing IgG was increased in the large and small intestines of mice that were i.p. immunized with Cry1Ac (Fig. 3), while the frequency of B cells expressing other antibody isotypes was not significantly affected.



Figure 2 Intraperitoneal immunization with Cry1Ac induces specific antibody cell responses in the lamina propria of the small and large intestine in Balb/c mice. Lamina propria lymphocytes were isolated from the small (SI) and large intestine (LI), and the elicited IgA, IgG, IgM and IgE anti Cry1Ac antibody cell responses were evaluated by ELISPOT as described in the Methods in control and immunized mice. Upper panel: Data shown are from Cry1Ac immunized mice and are expressed as the arithmetic mean  $\pm$  SD. (n = 5). In control mice were not detected significant anti Cry1Ac antibody cell responses, nor in the small neither in the large intestines; as the values recorded in this group for any isotype analysed were <2 spots per million of cells added (data not shown). Bottom panel: representative images of the spots recorded in immunized mice indicating the isotype and magnification. Control image corresponds to anti Cry1Ac IgG response in an un-immunized mouse.

Figure 3 FACS analysis of intestinal B lymphocytes expressing surface immunoglobulins from control and mice i.p. immunized with Cry1Ac. Single cell suspensions of isolated intestinal lymphocytes  $(1 \times 10^6 \text{ cells/sample})$ were stained with either FITC or Percp -anti-CD3, and either Percp or PE-anti-B220, and PE anti-IgG or IgM, and FITC anti IgA and IgE. Left: small intestine, Right: large intestine, upper panel: intraepithelial lymphocytes, Bottom panel: lamina propria. Data represent the mean percentage of cells expressing Igs among gated B lymphocytes ± SD from five independent experiments using cells of a given tissue from three mice.



### Some intestinal epithelial cells display an IgG positive reaction, and immunization with Cry1Ac increases its frequency

When we examined IgG expression in intestinal sections by immunohistochemistry, we observed some IgG+ lymphocytes located mainly in the lamina propria of the small and large intestines (Fig. 4), whose proportion appeared higher in the intestines of Cry1Ac-immunized mice. Interestingly, we observed that some epithelial cells also displayed IgG staining, suggesting that IgG might be being transported through the epithelia of both untreated and immunized mice (Fig. 4). To estimate the frequency of epithelial cells presenting IgG in the entire intestine, we isolated epithelial cells from the small and large intestines of control and immunized mice and performed flow cytometry analysis using E-cadherin as an epithelial cell marker. Cells were gated using an epithelial gate (based on forward and side scatter parameters). We distinguished two populations of epithelial cells based on their expression of E-cadherin (low and high) especially in the small intestine. Interestingly, we found that mainly in the large intestine the frequency of intestinal epithelial cells displaying IgG was higher in immunized than in control unimmunized mice (Fig. 5).

# In adult Cry1Ac immunized mice FcRn is expressed in intestinal epithelia

Although, it has been previously described that FcRn is developmentally downregulated in the rodent intestine



Figure 4 Immunohistochemical staining for detection of IgG in paraffin sections from the small and large intestine, of unimmunized and Cry1Ac immunized mice. Above: small intestine (SI), Bottom: large intestine (LI). Left: unimmunized, middle: i.p. immunized with Cry1Ac (Cry1Ac) and right: isotype control. In the small intestine IgG staining was detected in some cells located in the lamina propria as well as in several epithelial cells. The number of IgG positive cells observed in immunized mice was higher than in unimmunized mice. In the epithelia of the large intestine, IgG reaction was slight. Five micron thickness sections were counter stained with Harris's Haematoxilin. Each section represents consensus staining profiles from five independent animals. Arrow indicates an IgG + intraephitelial lymphocyte.





Figure 6 Immunization with Cry1Ac increases FcRn mRNA abundance in intestinal epithelial cells of adult mice. Epithelial cells from small and large intestine (SI and LI) from untreated neonate or adult mice; and adult Cry1Ac immunized mice (adult+Cry), were isolated and FcRn mRNA expression was analized by RT-PCR. Data represent mean ± SEM of relative expression of FcRn regarding GAPDH in three independent experiments.

Figure 5 Flow cytometry analysis of the intestinal epithelial cells displaying IgG. Cells were analysed within the epithelial gate and E-cadherin was used an epithelial cell marker. The frequencies of epithelial cells displaying IgG were higher in both the small and large intestines of immunized mice than in control mice, although the difference was markedly in the large intestine. Numbers represent the percentage of IgG positive cells  $\pm$  SD (from the marker to the right M1) within the epithelial gate. Eight independent experiments were performed using two mice per group and analysing the cells of a given tissue from individual mice. A representative histogram is shown.

and it is not expressed in the intestinal epithelia of adult mice, it has not previously tested whether its expression was induced upon intestinal IgG induction. As expected we detected high level of FcRn mRNA expression in neonatal intestines while in adult mice we did not detect its expression in intestinal epithelia of control un-immunized mice. Interestingly, we found that upon immunization with Cry1Ac adult mice displayed FcRn mRNA expression in intestinal epithelial cells, which was higher in the large intestine than in the small intestine (Fig. 6). Finally, to verify if the protein FcRn was expressed at higher levels in the intestinal epithelia of adult immunized than in adult unimmunized mice, the detection of the protein FcRn was determined by flow cytometry and immunofluorescence. The flow cytometry results shown in (Fig. 7) indicate that the percentage of epithelial cells expressing FcRn was significantly increased in immunized mice, moreover the expression of this protein was higher in the large intestine. Finally, we confirmed by immunofluorescence that the intestinal epithelia of neonatal mice presented the highest expression levels of FcRn, mainly in the small intestine (Fig. 8). While the intestinal epithelia of immunized mice presented increased expression of FcRn in relation to that found in control adult mice, especially in the large intestine. These results also confirmed that the intestinal epithelia of adult mice display a high IgG mark, although at lower levels than neonatal mice. In addition, we found that the co-localization of IgG and FcRn is much higher in the intestines on neonates than in adult mice.

## Cry1Ac increases FcRn expression in epithelial intestinal cells stimulated *in vitro*

To test whether Cry1Ac exerted a direct effect in epithelial cells on FcRn expression, we isolated epithelial cells from the small and large intestines from control mice and then we stimulated them in vitro with Cry1Ac protoxin. In freshly isolated cells that were incubated in RPMI medium for 3 h with or without Cry1Ac we found that FcRn is not expressed (Fig. 9 left). In marked contrast we found that stimulation with Cry1Ac for 24 h increased FcRn expression in epithelial cells from the small and large intestines, in comparison with cells incubated with medium alone. This increment induced by Cry1Ac was seen, in similar magnitude, either in cells freshly isolated cultivated for 24 h (data not shown), or in those maintained in culture for 7 days, and then stimulated with Cry1Ac for the last 24 h (Fig. 9 right). However, it is important to point out that unexpectedly we observed that after 24 h of culture, FcRn expression was also induced in cells cultured with medium alone.



Figure 7 Flow cytometry analysis of the intestinal epithelial cells expressing FcRn from control and immunized adult mice. Cells were analysed within the epithelial gate and E-cadherin was used an epithelial cell marker. The frequencies of epithelial cells displaying FcRn were higher in both the small and large intestines of immunized mice (Cry1Ac) than in control unimmunized mice (Control), although the expression was higher in the large than in the small intestine. Numbers represent the mean percentage of cells expressing FcRn  $\pm$  SD (from the marker to the right M1), from three independent experiments using two mice per group and analysing the cells of a given tissue from individual mice. A representative histogram is shown.

#### Discussion

Considering that we have previously described that Cry1Ac protoxin preferentially induces high specific IgG responses in intestinal fluids when it is intraperitoneally administered to mice [22], we used the same protocol in the present study as a model, to characterize the intestinal antibody response, as well as to determine whether upon intestinal IgG induction, the expression of FcRn was induced in intestinal epithelial cells, in adult mice.

The results shown in the present study indicate that intraperitoneal immunization with Cry1Ac protoxin induces significant intestinal IgG cell responses accompanied by an increased frequency of epithelial cells displaying localization of IgG. Moreover, we found that in adult immunized mice FcRn expression was detected in intestinal epithelial cells while in unimmunized mice the expression was undetectable.



Figure 8 Localization of IgG and FcRn in intestines from neonatal and adult unimmunized and immunized mice. Immunofluorescence in labelled intestinal sections from neonatal (neonate), adult control (unimmunized) and adult immunized mice (Cry1Ac i.p.), was visualized using a confocal microscope. Intestinal tissue sections were double labelled for; Left: IgG (green) and Middle: FcRn (blue). Right: merged images (clear blue). Double staining (Merge) shows the bright and wide co-localization of IgG and FcRn observed in neonatal intestines (especially in the small intestine), in comparison with that found in tissues from adult mice. In immunized mice the localization of both IgG and FcRn is increased in the epithelia in relation to adult unimmunized mice, which presented scarce FcRn epithelial mark and moderate IgG staining. Each figure is representative of three similar experiments. Control staining with isotype resulted in no detectable staining (not shown).

In addition to IgA, which is the predominant immunoglobulin present in the majority of secretions, quite significant quantities of IgG can also be detected within the mucosal secretions of adult human and rodents that, in certain locations may reach levels approximating that observed for sIgA, or even higher [30–32]. Secretory IgA (sIgA) is well-known to be transported across epithelial cells into the lumen through an active unidirectional receptor-mediated process known as transcytosis by the polymeric Ig receptor (pIgR) [6]. Conversely, the mechanisms by which IgG reaches the luminal intestinal secretions in adult mice are not entirely established. IgG is



thought to enter various external secretions by receptorindependent paracellular diffusion, receptor (FcRn)-mediated transepithelial transport, and fluid-phase endocytosis depending on the tissue involved [1]. Based on the relative proportions of the IgG subclasses as well as the ratio of specific versus total IgG detected in external secretions and plasma, it has been assumed that the presence of IgG antibodies in external mucosal secretions is originated exclusively or predominantly from the circulation through transudation [33, 34]. However, a few studies have shown evidence suggesting that the source of IgG found in secretions might arise from local mucosal production. For example, it has been demonstrated that the specificity of IgG Abs in secretions may differ from that of their serum counterparts and vary depending on the mucosal compartment [35]. Moreover, female genital tract secretions contain a significant proportion of IgG, which seems to be locally produced by abundant IgGproducing cells [33]. Likewise, present results indicate that in the intestinal lamina propria of the small and large intestine, of Cry1Ac immunized mice are induced significant specific IgG-producing cell responses. Although the number of IgG-producing cells was not higher than those producing other isotypes, they appear to produce a greater amount of antibody.

Induction of specific IgG cell responses in the intestinal lamina propria has also been reported after infection or immunization. Indeed, following inoculation with virulent rotavirus, the numbers of IgG antibody secreting cells (ASC) induced were comparable to those of IgA ASC in the intestinal lamina propria [36]. After a virulent enteric coronavirus challenge [37], smaller numbers of IgA ASC and larger numbers of IgG ASC were

Figure 9 Flow cytometry analysis of FcRn expression in intestinal epithelial cells stimulated in vitro with Cry1Ac. Epithelial cells were isolated from the small (SI) and large (LI) intestines from three control adult Balb/c mice and cultured in supplemented RPMI 1640 medium. Cells were cultured in 24 well plates  $(2 \times 10^6 \text{ cells/ml})$  in culture medium with or without Cry1Ac (20  $\mu$ g/ml) at 37 °C in 5% CO2 for 3 h in freshly isolated cells (left) and after the last 24 h in cells cultivated for 7 days (right). The cells were harvested and stained with anti-FcRn and E-cadherin antibodies and analysed by flow cytometry as described in Fig. 7. Above: small intestine (SI), Bottom: large intestine (LI). Numbers represent the mean percentage of cells expressing FcRn ± SD (from the marker to the right M1), from two independent experiments using the isolated cells of a given tissue from individual mice (n = 3). Representative histograms are shown.

observed in the intestines of pigs. It has been demonstrated that specific mucosal IgG responses can be induced (in extracts obtained from various effectors sites: the jejunum, ileum, cecum, colon, salivary glands, and uterus-vagina) by adjuvant-free parenteral immunization with soluble antigens [38]. Consistently with the present results several reports also have found that intraperitoneal immunization can induce humoral and/or cellular immune responses both at the systemic and mucosal intestinal levels [28, 38–40].

Using flow cytometry analysis we determined that the intestinal lamina propria contained IgG+ lymphocytes whose frequency was increased upon immunization. Therefore, our results indicated that there was local production of specific IgG by lamina propria cells as well as increased frequencies of IgG+ B cells in the lamina propria of immunized mice. However, the possibility that part of the high IgG responses found in intestinal secretions might have come from the serum as a result of an intestinal inflammatory reaction provoked by immunization, could not be excluded. Thus, we considered important to determine if the immunization provoked intestinal inflammation as well as to evaluate the presence of intestinal IgG+ lymphocytes by immunohistochemistry. We did not find any evidence of an inflammatory reaction or tissue damage upon histological examination of the intestines of immunized mice. While the immunohistochemical examination confirmed, the presence of IgG+ lymphocytes located in the lamina propria of both, the small and large intestines, of control and immunized. Interestingly, we also observed that some epithelial cells displayed IgG staining, (at both the apical and basal regions), suggesting that IgG was being transported

through these cells instead of being delivered to the lumen either by intercellular transudation or by the leakage of tight junctions that could be provoked by an immunization-induced inflammatory reaction. Indeed, the intestinal IgG reaction was detected in B cells located in the lamina propria, within blood vessels, and within epithelial cells, but not between epithelial cells.

It is well established that the neonatal Fc receptor for IgG (FcRn) is the receptor responsible for mediating IgG transport across epithelial barriers. However, the evidence showing that FcRn is not expressed in the intestinal epithelia of adult mice [7, 10] had suggested that this receptor only participate in intestinal IgG transport in neonatal mice, while in adult rodents, this transport was not mediated by this receptor. However, it has not been examined before whether FcRn was expressed in intestinal epithelia upon intestinal IgG induction. Importantly, present results indicate that the expression of this receptor can be reactivated to some extent in adult mice. Although the expression of FcRn achieved following immunization with Cry in adult mice was substantially inferior in comparison with that found in neonatal intestines, probably FcRn might facilitate epithelial IgG transport in the intestine of adult mice exhibiting intestinal IgG responses, since immunized mice also showed increased numbers of epithelial cells containing IgG.

On the other hand, the increment on FcRn expression in epithelial cells induced in immunized mice was more pronounced in the large than in the small intestine further supporting the described striking differences in phenotype and function between both intestines [26].

We presume that the observed increase in FcRn expression in intestinal epithelia of adult immunized mice was provoked as a consequence of the intestinal IgG immune response induced, rather than by any possible direct effect of the protein exerted on mammalian epithelial cells, given that the antigen may has acted, on a distinct cellular type, such as macrophages or dendritic cells, before reaching the epithelia, as it was administered intraperitoneally. However, when the effect of stimulation with Cry1Ac was tested in vitro in isolated epithelial intestinal cells, we found an increase on FcRn expression (after 24 h), suggesting that the protein might also exerts direct effects in intestinal mammalian epithelial cells, perhaps throughout an unknown receptor as has been previously suggested. Indeed, despite Cry proteins are not toxic for vertebrates and Cry1Ac toxin is known to form pores exclusively in lepidopteran insect larvae midgut epithelial cells, it has been shown that Cry1Ac protoxin binds to brush border membranes vesicles prepared from mouse small intestine in vitro, and induces a transient hyperpolarization of the jejunum [41]. Although, the nature of the interacting molecules with Cry proteins in mammalian enterocytes seems to be different to the receptor glycoproteins

described in insects, like the 120 kDa aminopeptidase N [18] and the 210 kDa cadherin-like glycoprotein (Bt-R1) [42], since binding was not inhibited by the GalNAc, mannose or biotin.

Further detailed studies are still required to determine the mechanisms leading to the observed *in vivo* and *in vitro* effects induced by Cry1Ac in epithelial cells. It also would be interesting to determine if immunization with additional antigens other than Cry1Ac, also provoke increased intestinal IgG and FcRn expression in epithelium. In addition, it remains to be determined which were the factors leading to the induction in FcRn expression observed in intestinal epithelial cells cultured with medium alone.

Based on our previous studies, showing that the Cry1Ac protoxin is a potent mucosal and systemic immunogen and adjuvant [20-22] we have suggested that this protein may be a valuable tool to improve mucosal vaccines, while the present data suggest that the Cry1Ac protoxin may also be useful to characterize the induction of intestinal IgG responses. This study topic is important since like IgA, mucosally associated IgG has been shown to contribute importantly to mucosal host defense [3, 9]. Indeed some reports have also shown that passive administration of neutralizing IgGs can prevent mucosal human immunodeficiency virus transmission in rhesus macaques or macaque neonates [3]. Likewise, virus-specific IgG in vaginal secretions of immune mice significantly reduces infection of the vaginal epithelium by challenge with herpes simplex virus type 2, whereas S-IgA contributes relatively little to this protection. Moreover, it has been proposed that FcRn regulates mucosal IgG immune responses to luminal bacteria, as selective expression of FcRn in the epithelium, (using a transgenic mice model expressing FcRn beyond the neonatal period of life), was shown to be associated with secretion of IgG into the lumen that allows for defense against an epithelium-associated pathogen (Citrobacter rodentium) [9].

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